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Symposium on
Nutritional Aspects of Blood Formation*

Hematology and Nutrition have many common bonds. The blood and bone marrow provide a readily available laboratory in which the nutritionist may observe the effects of various nutrients on cellular growth and development. Likewise, the hematologist depends upon many of these nutrients to relieve disorders of the blood which result in anemia. So it is not surprising that each of these two fields depends so much on the other. The nutritional anemias are the common meeting ground.

For many years, these anemias have been one of the major research interests of the laboratory of hematology and nutrition, Department of Medicine, University of Cincinnati College of Medicine. It is very gratifying that the National Vitamin Foundation asked us to organize this symposium on the Nutritional Aspects of Blood Formation. A very distinguished panel of speakers has gathered here to bring together and discuss pertinent information on how and why the cells of the bone marrow grow and develop into the cells which circulate in the blood stream. A symposium of this kind gives all who are interested the opportunity to view the broad aspects of the field as well as to derive new ideas from a synthesis of the older ones. Cincinnati and its university are honored to have been chosen for this very significant meeting.

RICHARD W. VILTER, M.D.
Chairman

* Held under the auspices of the National Vitamin Foundation, at the University of Cincinnati, October 22, 1954.

The Importance of Nutritional Factors *in the Pathogenesis of* Iron-Deficiency Anemia

By CARL V. MOORE, M.D.*

STUDENTS of iron metabolism in the United States, with extremely few exceptions, are agreed that nutrition plays a relatively minor role in the production of iron-deficiency anemia among adults unless blood loss also occurs. It is argued: (a) that iron is excreted in such small amounts that a positive balance is maintained even when the diet is deficient or absorption defective; and (b) that poor nutrition leads to the development of iron deficiency only when requirements are increased by growth, as during infancy and childhood, or by chronic hemorrhage. With poor diet or poor absorption, even the amount of blood lost with normal menstruation is enough to lead gradually to iron deficiency. This belief is strengthened by careful clinical study. Patients with iron-deficiency anemia are not infrequently seen who give no history of chronic hemorrhage or in whom no source of blood loss can at first be discovered. But when one persists—and sometimes weeks or months of observation are required—one can almost invariably demonstrate eventually that occult hemorrhage is occurring intermittently from the gastrointestinal or urinary tracts.

The above concept, however, is not shared by workers in many other parts of the world. They claim that when diets are inadequate for long periods of time, either because of disturbed food production during war or because of persisting food shortages in the most heavily populated areas, iron deficiency develops in adults on the basis of poor nutrition alone. There have been so few data on the absorption of iron from foods and on iron excretion in man that it has been difficult to resolve this difference of opinion, but, in an attempt to do so, Doctor Reubenia Dubach and I have been collecting some of the missing information. On the basis of our experiments, to be reported in detail elsewhere, I should like to analyze the importance of nutritional factors in the production of iron-deficiency anemia.

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ABSORPTION OF IRON FROM FOODS

Most of the information about iron absorption is of little nutritional significance, since it has been obtained from experiments in which inorganic iron salts were fed to patients or animals. The ionizable iron in many foods has been measured on the assumption that only this portion is available for absorption, but when balance studies are done on patients in an attempt to determine the amount retained, the technical difficulties become enormous. Diet and water intake must be rigidly controlled. A relatively large error is involved in the chemical determination of iron in feces, and it is impossible to differentiate between unabsorbed and excreted iron. The few meticulous studies that have been done indicate that from spinach or from mixed diets the per cent of iron absorbed varies from about 11 to 14 per cent, while from beef the absorption may be as high as 21 per cent.[1]

In order to circumvent the difficulties involved in balance studies, we have incorporated radioactive iron into various foods and measured absorption under a variety of conditions with the radioactive technique.[2,3] Radioactive iron has been injected into hens so that the eggs produced contain the isotope. After several weeks, the hens have been killed so

that their livers and muscle might also be fed. Vegetables have been grown in nutrient solutions to which radioiron was added; they then had a portion of their iron in isotopic form. Foods have been cooked, or prepared as they would be in a normal American diet and fed to fasting subjects. In most experiments, the amount of radioiron incorporated into circulating hemoglobin has been used as the measure of the quantity absorbed, but in some instances the unabsorbed portion recovered in the feces has also been determined. Subjects, for the most part, have been normal healthy students or laboratory personnel, and patients with iron-deficiency anemia.

The most important observation has been that, with few exceptions, the amount of iron absorbed from foods by normal subjects has been 10 per cent or less (Fig. 1). In only two instances was absorption significantly greater than 10 per cent; chicken muscle had been fed in both cases. We next attempted to find out

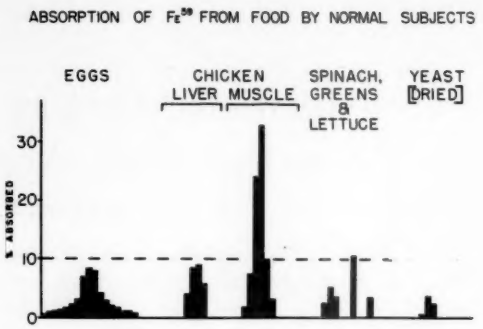

Fig. 1. Each column represents a separate experiment. The dotted horizontal line at the 10 per cent level helps to emphasize the fact that very few subjects absorbed more than this amount.

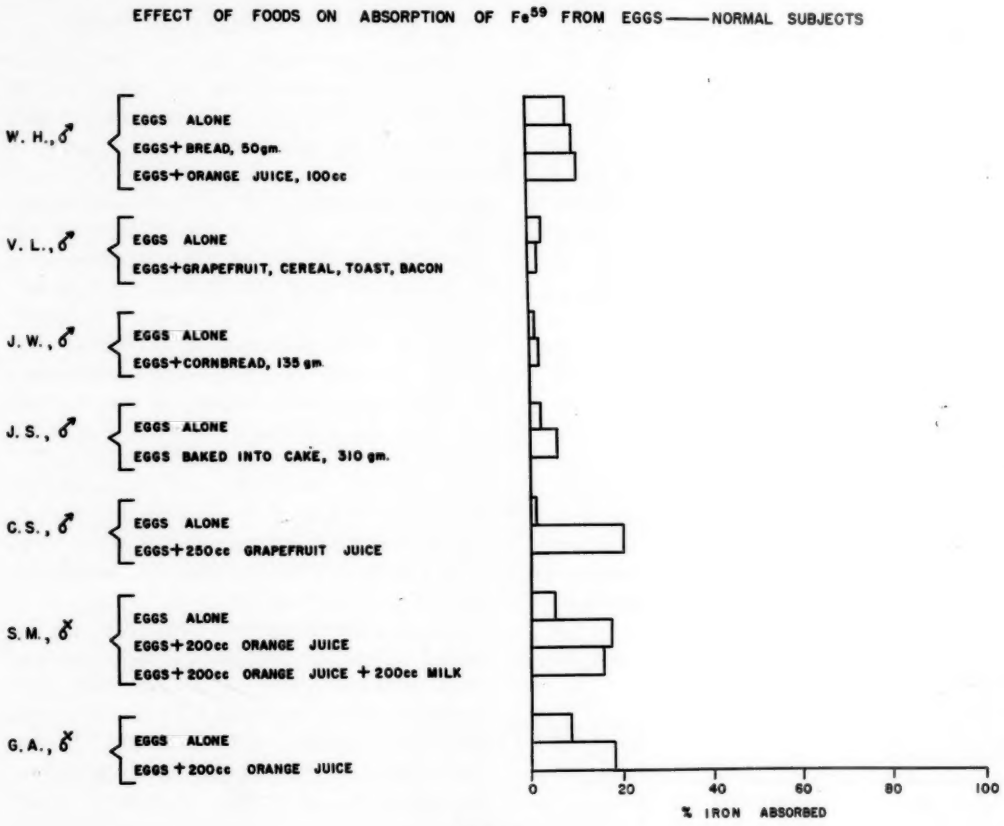

Figure 2.

whether patients with iron-deficiency anemia retained the iron from foods any more efficiently. When eggs were fed, absorption was greater than 10 per cent in only two out of ten experiments. In the few studies so far completed with chicken liver, vegetables, and yeast, however, iron-deficient patients retained more than 10 per cent in the majority of instances.

the ascorbic acid contained in fruit might increase absorption by promoting the reduction of ferric iron in food to the ferrous form. Crystalline ascorbic acid in comparable amounts had a similar effect; when the amount of ascorbic acid was increased to one gram, there was even greater absorption (Fig. 3). Further studies have indicated that: (a) ascorbic acid usually increases the assimilation of food iron

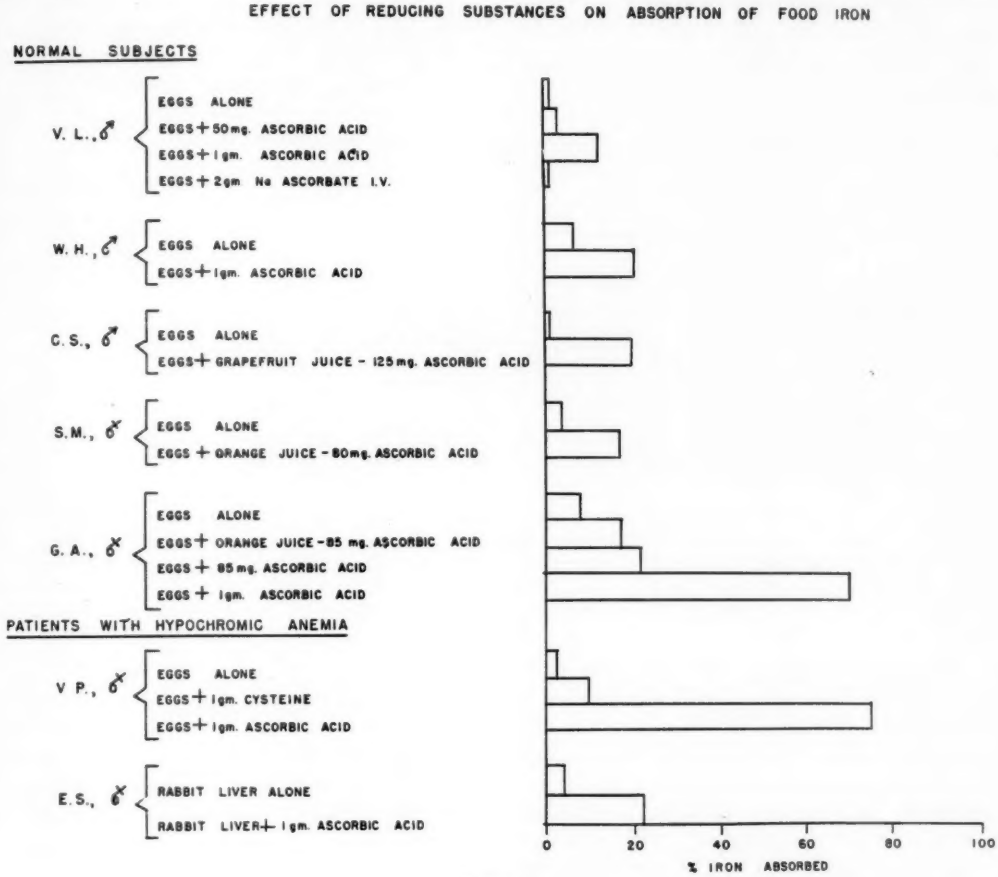

Figure 3.

The effect of other foods on the absorption of iron from eggs has also been measured (Fig. 2). The eggs were baked into cake or corn bread, or scrambled and fed along with toast, bacon, cereal, and fruit juice. Greater assimilation could be detected only with relatively large amounts (200 to 250 cc.) of citrus fruit juices. That observation suggested that

even more in iron-deficient than in normal subjects; (b) reducing substances such as cysteine have a similar action; while (c) other organic acids (citric, lactic, tartaric) are without effect.

A preliminary attempt has also been made to evaluate the importance of gastric hydrochloric acid on the absorption of iron from

food. For many years it has been thought that individuals with hypochlorhydria or achlorhydria probably assimilate food iron poorly. In such patients, however, we have not been able to increase absorption of the radioiron by adding 60 cc. of 0.1 *N* HCl to cooked eggs, or by adding enough 1 *N* HCl to reduce the pH of the mixture to 1.5 before it was given by stomach tube. On the other hand, ascorbic acid in a dose of 250 to 1000 mg. did increase absorption very significantly, even though it had comparatively little effect on gastric acidity.

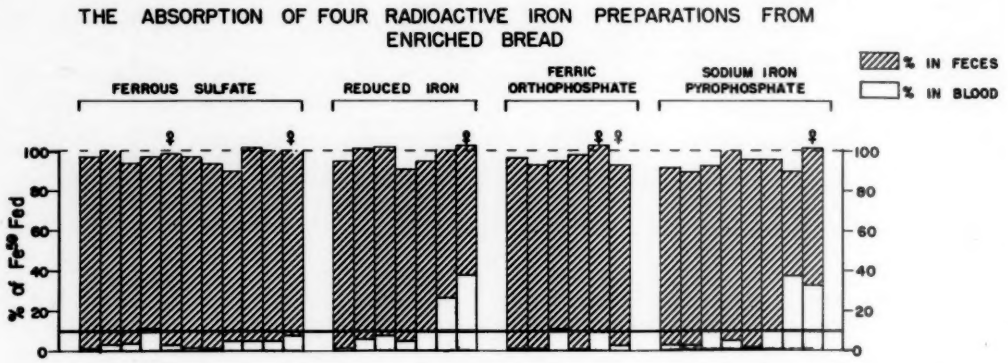

Fig. 4. The subjects were healthy medical students. Each ate four slices of bread enriched with one of the radioactive iron preparations. In many of the experiments, the sum of the radioactivity in the blood and that recovered from feces does not equal 100 per cent. The unaccounted for portion represents a combination of the following three factors: absorbed iron not synthesized into hemoglobin, error of determination, and failure of the subject to make absolutely quantitative collection of feces.

Of related interest are observations recently completed on the absorption of iron from bread baked with flour which had been enriched with radioactive iron.[4] The four iron preparations used most commonly in the enrichment program (ferrous sulfate, reduced iron, ferric orthophosphate, and sodium ferric pyrophosphate) were added to flour and baked into bread under conditions which closely similated those employed in the baking industry. The amount absorbed by 28 of 32 healthy young men and women varied from 1 to 12 per cent (Fig. 4). The remaining four subjects retained from 26 to 38 per cent of the radioactivity fed, but there was reason to suspect that their iron stores were suboptimal in each instance. The only three patients with iron-deficiency anemia included in the study assimilated 45 to 64 per cent of the iron taken. The addition of 1 gram of ascorbic acid to the bread eaten by six of the healthy subjects increased the absorption of iron two to three times.

From the above observations, one may conclude that the food iron absorbed by healthy men and women probably does not average more than about 10 per cent of that in the diet, and in many individuals the amount is probably less. The diet of adults in the United States contains approximately 12 to 15 mg. of iron per day. The amount of iron absorbed from food per day, therefore, probably varies from about 0.6 to 1.5 mg. Poor diet, infection, diarrhea, or steatorrhea[5] would decrease this amount even further, while iron deficiency would probably result in some increase.

Excretion of Iron

Because conservation of iron is so tenacious and because the amount excreted is so small, many people have mistakenly assumed that no iron whatever is lost from the body except as shed blood. The error of this assumption becomes obvious from the following considerations. All cells in the body contain iron. When leukocytes and epithelial cells are dis-

charged in body secretions, when erythrocytes appear in urine, when cells are desquamated from the skin or the mucosa of the intestinal tract, and even when hair grows, some iron is lost. Attempts to estimate this amount and to determine in addition how much is excreted in other ways has proved a very difficult task. The metal is so ubiquitous that it has been almost impossible to differentiate between ex-

THE AVERAGE DAILY RADIOIRON EXCRETION AND THE CALCULATED TOTAL IRON EXCRETION OF TEN HUMAN SUBJECTS

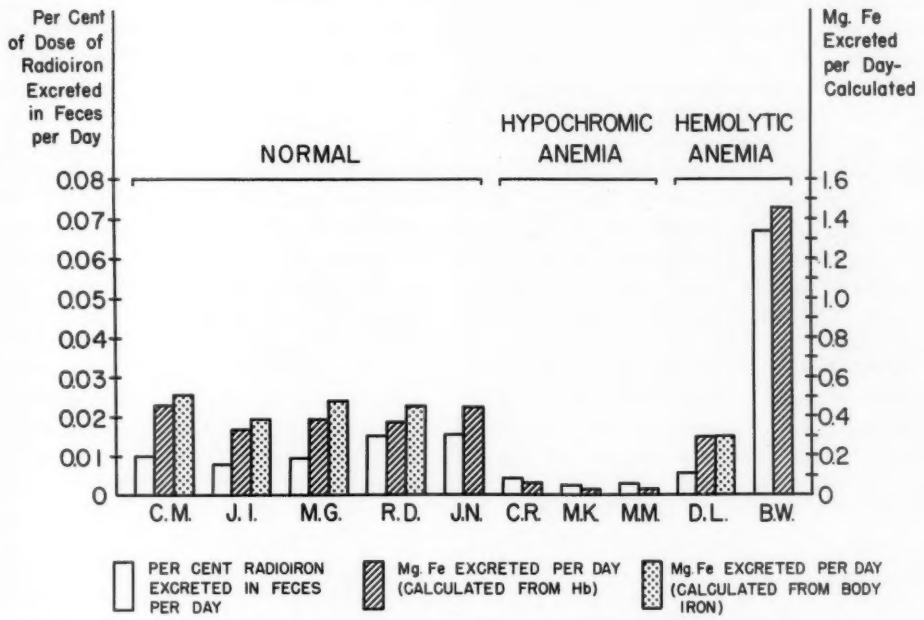

Fig. 5. (Reproduced by permission of the *Journal of Laboratory and Clinical Medicine.*)

creted iron and that present because of contamination or because it wasn't absorbed. Radioiron has provided a partial, but not a complete, solution to the problem.

The isotope has been injected intravenously in tracer amounts into normal subjects and patients with hypochromic or hemolytic anemias.[6] Five-day fecal collections have then been made at intervals up to 150 days; this long period was used because most of the injected radioiron was promptly synthesized into hemoglobin and we wanted the collection periods to be greater than the 120-day life span of the erythrocytes. Every fecal specimen on every subject contained a detectable amount of radioactivity; the amount averaged 0.01 per cent of the dose per day for normal subjects (Fig. 5). Three iron-deficient patients excreted much smaller amounts, while one young woman with a hemolytic (sickle cell) anemia excreted more. No significant increase in fecal radioactivity was detected at or near the 120th day after the injection had been given. It is difficult, however, to calculate from these figures what the fecal excretion of all body iron—inert as well as radioactive—would be. Two calculations were made: one from the ratio of total hemoglobin iron to radioactive hemoglobin iron; the other from the ratio of estimated total body iron to the injected dose of the isotope (Fig. 5). Fecal excretion calculated in these ways for normal subjects varied from 0.3 to 0.5 mg. per day. For iron-deficient patients the value was about one-tenth as much. While these cal-

culated values are admittedly estimations, one can be reasonably certain that they do not vary from true figures by more than 100 per cent. One cannot differentiate with this method among the following three possible sources of fecal iron: (a) true excretion; (b) desquamated mucosal cells; and (c) iron delivered to the duodenum via the bile and not completely absorbed.

Some of the injected radioiron was also found regularly in sweat for at least 320 days, the longest time after administration that a collection was made. The amount was small, but definitely measurable. We were not able to determine whether it came from sweat glands by a process of true excretion or from desquamated epithelial cells. Calculations indicated that under normal conditions the total iron lost from dermal surfaces is certainly less than 1 mg. per day and probably not more than 0.5 mg. It is of interest that radioactivity could also be detected in hair clippings obtained several months after the injection of radioiron and washed with dilute HCl to free them from surface (sweat) contamination.

To the above values must be added the small amount of iron found in urine. From these figures, one can estimate that the adult male loses or excretes between 0.5 and 1.5 mg. of iron per day; the median value of 1 mg. is probably not in error by more than 20 to 25 per cent. Menstrual blood flow of 35 to 70 cc. every 28 days in a normal woman with a hemoglobin value of 12 grams would account for an additional average loss of 0.5 to 1 mg. per day.

Importance of Nutrition in the Pathogenesis of Iron-Deficiency Anemias

Even though the data presented for the absorption of iron from food and for the excretion of iron are admittedly incomplete and provide approximations only, they do permit a better evaluation of the importance of nutritional factors in the pathogenesis of iron deficiency than has previously been possible. If the adult male absorbs an average of 10 per cent of the iron in a diet that contains 12 to 15 mg. per day, he retains 1.2 to 1.5 mg. Since he excretes only about 1 mg. or less of iron per day, he maintains a positive balance rather easily. The adult woman, however, during the years of menstruation and child bearing tends to eat less and loses additional amounts. A mother furnishes her fetus during gestation with about 300 to 500 mg. of iron, or between 1 and 2 mg. per day throughout the duration of her pregnancy. The volume of menstrual blood is normally approximately 35 to 70 cc. If the hemoglobin value of that blood is 12 grams per 100 cc., then 14 to 28 mg. of iron would be involved. Spread evenly over a 28-day menstrual cycle, this equals 0.5 to 1 mg. per day. Iron balance in a young woman, therefore, is precarious, so that poor diet or poor absorption may lead to iron deficiency even though menstrual loss remains normal. Frequent pregnancies or any increase in menstrual flow make her all the more susceptible. Almost nothing is known about iron excretion in children, but the infant and the growing child need iron to increase their blood volume, their myoglobin volume, and the respiratory enzymes required by all cells. Since the body of an infant contains about 0.5 gram and that of an adult 3 to 5 grams of iron, there must be a net gain during the first twenty years of life of 2.5 to 4.5 grams; this net gain averages 0.12 to 0.22 gram per year or about 0.35 to 0.6 mg. per day. In all probability, therefore, the positive iron balance maintained by normal children during their most active growth must be slight, so that poor diet or poor absorption could readily produce iron deficiency.

It is much more difficult, however, for iron deficiency to develop in the adult male or the post-menopausal woman on a purely nutritional basis without any associated blood loss. The calculations in Table I, based on two hypothetical patients, demonstrate that if these two persons excreted 1 mg. of iron per day and absorbed none at all, six and four years, respectively, would be required before they would become deficient enough to have only 7.5 grams of hemoglobin per 100 cc. These figures ignore the evidence that as patients become iron-deficient they excrete smaller amounts of the metal. Any iron contained in

TABLE I.

Calculation of Time Required for Development of Iron Deficiency on Nutritional Grounds Alone in Two Hypothetical Patients (see text)

	Adult male Hb. 15 Gm./100 cc. Bl. vol. 5000 cc.		Post-menopausal woman Hb. 14 Gm./100 cc. Bl. vol. 4000 cc.	
A. When Normal				
Total Hb. iron	2500 mg.		1900 mg.	
Storage iron	1000 mg.		500 mg.	
Total		3500 mg.		2400 mg.
B. After development of Fe deficiency (Hb. of 7.5 Gm./100 cc.)				
Total Hb. iron	1250 mg.		950 mg.	
Storage iron	0		0 mg.	
Total		1250 mg.		950 mg.
Deficit in Hb. and storage iron (with this degree of hypochromic anemia)		2250 mg.		1450 mg.
Time required to produce deficiency if no iron is absorbed and 1 mg. excreted per day		2250 days (6.3 yrs.)		1450 days (4 yrs.)

the deficient diet, furthermore, would tend to be assimilated with greater than normal efficiency unless there were a serious absorptive defect in the intestinal tract. Both factors would considerably delay the appearance of iron-deficiency anemia. One is forced to admit, however, the theoretical possibility that men or post-menopausal women who consume very deficient diets or who have absorptive defects could, over a period of many years, develop iron deficiency on a nutritional basis alone. On the other hand, it seems far more likely that patients with inadequate diets or poor absorption also lose small amounts of blood that go undetected. In this country, at least, there is not a single, well-documented published instance in which iron-deficiency anemia has been shown to develop in an adult in the absence of blood loss. The bleeding may be slight and intermittent, so that diligent search is required for its detection. With such hemorrhage, even though small in amount, inadequate iron intake or absorption then becomes of major importance in the pathogenesis of hypochromic anemia.

Summary

Nutritional factors are of major importance in the production or prevention of iron-deficiency anemia. Healthy persons probably maintain a positive iron balance by a narrower margin than was formerly believed. Approximately 5 to 10 per cent food iron seems to be assimilated by normal adults; daily retention on a diet containing 12 to 15 mg. of iron, therefore, may be estimated to be about 0.6 to 1.5 mg. The amount of iron lost from the body each day, in all ways except as blood, seems to be between 0.5 and 1 mg. The added requirements of children and young women to compensate for growth needs and menstrual flow place them in a precarious state of iron balance, so that poor diet or poor absorption can readily lead to the production of hypochromic anemia. In adult men or post-menopausal women, however, nutritional factors appear to be of less importance in the pathogenesis of iron deficiency. If purely nutritional iron deficiency ever occurs in these people, many years would be required for its production. It is more likely that occult, intermittent bleeding, often difficult to detect, must also be present along with inadequate diet or malabsorption before severe degrees of iron deficiency develop.

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The Relationship of Copper, Cobalt, *and Other* Trace Elements *to* Hemopoiesis

By GEORGE E. CARTWRIGHT, M.D.*

IT IS possible and even likely that many, if not most, of the so-called "trace elements" are essential for hemopoiesis. However, because they are required in only small quantities, naturally occurring deficiencies are rarely, if ever, observed in human subjects and it is therefore difficult to prove that they are essential. This is true even in experimental animals raised under the most rigorous circumstances. In general, these facts have not been well understood by clinicians, and the trace elements have been used rather widely as therapeutic agents. Only a few individuals have distinguished clearly between the physiological importance of trace elements and their lack of importance as a supplement to natural diets for human and animal consumption.

The presence of iron, copper, cobalt, zinc, manganese, magnesium, molybdenum, and possibly titanium and vanadium has been demonstrated in bone marrow, or in the cells of the circulation, or in both. The presence of an element in hemopoietic tissue implies that it is there to serve a useful and essential function. Unfortunately, in the case of many of these elements, so little is known that nothing can be said concerning their function in relation to hemopoiesis. This field, at least from the standpoint of total accumulated knowledge, is in its infancy.

The purpose of this presentation is to summarize and review briefly our present knowledge of the physiological role of several elements in hemopoiesis and to comment on the clinical application of this knowledge. For bibliographical references, several reviews are available.[1-5]

COPPER

Physiologic Considerations

A deficiency of copper in most, if not all mammalian species, is associated with a severe anemia. In swine, the anemia which develops resembles closely the anemia associated with iron deficiency (Table I). The erythrocytes are microcytic and hypochromic, and there is normoblastic hyperplasia of the bone marrow.

TABLE I

Copper Deficiency in Swine, Comparison with Iron Deficiency

Determination	Control	Copper-deficient	Iron-deficient
Volume packed red cells, ml./100 ml.	42	22	21
Mean corpuscular volume, cu. μ.	56	39	36
Mean corpuscular hemoglobin conc., %	33	29	28
R.B.C. copper, μg./100 ml.	110	67	110
Mean corpuscular copper, $\mu\mu\mu$g.	61	26	39
Plasma copper, μg./100 ml.	186	15	207
Plasma iron, μg./100 ml.	175	38	30
Total iron-binding capacity, μg./100 ml.	511	628	864

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A marked hypoferremia develops early in the course of the deficiency, in spite of the fact that the copper-deficient swine receive 30 mg. of iron/Kg. of body weight daily from the beginning of the experiment. The level of iron in the plasma is reduced to levels comparable to those observed in iron-deficient swine and, as in true iron-deficiency, there is an increase in the iron-binding capacity of the plasma. The anemia and the accompanying biochemical alterations are rapidly and completely corrected by the administration of copper either orally or intravenously, but respond little or not at all to the oral or intravenous administration of large quantities of iron.

The manner in which copper functions in erythropoiesis is obscure. The morphological and biochemical similarities between copper and iron deficiency suggest that copper may be involved in the metabolism of iron. Studies on the metabolism of iron in copper-deficient swine indicate that such animals are unable to absorb iron at the normal rate. This is indicated by the fact that, in the absence of copper, the sum of the iron found in the blood, liver, kidney, spleen, and heart of animals, fed the same amount of iron for the same period of time as litter-mate controls, is reduced to levels comparable to those seen in iron-deficient swine. Similarly, the total amount of radioactive iron found in the tissues of copper-deficient swine following the oral administration of radioactive iron is significantly less than in control animals. Finally, copper-deficient swine respond to the administration of copper only when iron is included in the diet, thus indicating that the tissues do not contain sufficient iron for hemoglobin formation.

The defect in hemoglobin synthesis in copper deficiency cannot, however, be attributed solely to a failure to absorb iron, because the anemia is not relieved by the intravenous administration of large quantities of this element. This observation, in addition to those given above, led us in our earlier work to suggest that copper is concerned in some manner in the absorption, mobilization, and utilization of iron. More recent studies in our laboratory* (Table II), carried out with the use of tracer quantities of radioactive iron, indicate that in copper-deficient swine, both the plasma iron turnover rate and the amount of iron incorporated into red cells each day are increased above the normal, and that the erythrocyte survival time is decreased to about one-fifth of the normal value.

TABLE II

Ferrokinetic Studies in Control and Copper-Deficient Swine and in Swine with Hemolytic Anemia

Group	Control	Copper-deficient	Hemolytic anemia*
No. pigs	20	4	3
Plasma iron, μg./100 ml.	166	39	159
T ½ hr.†	1.2	0.5	0.3
Plasma iron turnover rate, mg./Kg./24 hr.	1.1	1.7	4.9
Injected dose incorporated into R.B.C., %	91	67	86
R.B.C. iron turnover rate, mg./Kg./24 hr.	0.6	1.1	4.1
Erythrocyte survival, days	61	13	5

* Induced with phenylhydrazine.

† Time required for one-half of the isotope to disappear from the plasma.

It is also apparent (Table II) that the bone marrow of copper-deficient swine is incapable of increasing its production to the same extent as that of animals in which a severe hemolytic anemia has been produced by the administration of phenylhydrazine. Thus, there is both a limitation in the rate of synthesis of cells and a decrease in the red cell survival time. This suggests that when a serious depletion of copper exists, defective cells, which are unable to survive the normal stresses placed on a cell in the circulation, are produced, and that, concomitantly, the rate of erythropoiesis cannot be increased sufficiently to compensate for the increased rate of destruction. Studies concerning the precise function of copper in erythropoiesis and in the circulating red cells are now in progress.

The copper compounds which are known to be present in mammalian tissue are listed in Table III.

Butyryl coenzyme A (CoA) dehydrogenase is a deep brilliant green cuproflavoprotein enzyme containing 1.2 per cent riboflavin and 0.345 per cent copper. This enzyme catalyzes

* J. A. Bush, W. N. Jensen, G. E. Cartwright, and M. M. Wintrobe: Unpublished data.

TABLE III

Copper-Containing Proteins and Enzymes in Mammalian Tissues

Protein	Mol. wt.	Cu	Substrate
		%	
Butyryl CoA dehydrogenase	120,000–220,000	0.35	Saturated Acyl CoA derivatives of fatty acids (C_3 to C_8)
Tyrosinase	100,000*	0.25	Tyrosine, DOPA
Ceruloplasmin	151,000	0.34	Paraphenylenediamine
Hepatocuprein	35,000	0.34	?
Erythrocuprein	?	?	?
Milk copper protein	?	0.19	?

* Values given are for tyrosinase obtained from plant source.

the first oxidative step in the conversion of lower fatty acids (C_3 to C_8) to acetyl CoA. Whether or not this enzyme is present in red cells is not known, and its importance in erythropoiesis, if any, has not been investigated.

The enzyme tyrosinase catalyzes both the oxidation of L-tyrosine to dihydroxyphenyl L-alanine (*dopa*) and the oxidation of dopa to melanin. Again, the importance of this enzyme to erythropoiesis is not understood and has not been investigated.

Ceruloplasmin is the blue, copper-containing α-2 globulin of plasma. Approximately 96 per cent of copper in normal plasma is bound to this protein. Ceruloplasmin has weak oxidase activity on the substrate paraphenylenediamine. Its natural substrate, if any, is not known. Hepatocuprein, a blue copper-containing protein, has been isolated from liver. Its function is unknown. The copper present in red cells is bound to protein, but this protein has not as yet been isolated. Studies in our laboratory by Dr. Harold Markowitz indicate that "erythrocuprein" is immunologically distinct from ceruloplasmin. Whether it is identical or not to hepatocuprein is not known. Little is known about the chemical nature and functions of the erythrocyte copper protein or proteins. No function has as yet been assigned to the milk copper protein.

Clinical Application

As yet, a clearly proved case of dietary copper deficiency has not been described in a human subject. This is understandable since a diet of even mediocre quality contains from 2.5 to 5.0 mg. of copper per day, and the daily adult requirement is approximately 2 mg. per day. Copper is widely distributed in foodstuffs and is readily stored in the body. In order for an adult to become depleted in copper, it would be necessary to consume a diet so low in calories that death due to caloric inadequacy would supervene long before a serious depletion of copper could result. Although both human and bovine milk are low in copper, and although it might be anticipated that the copper requirement of the rapidly growing infant would be high, this eventuality seems to have been anticipated by providing for high fetal stores of copper. These are 5 to 10 times higher than in the adult and are apparently sufficient to compensate for the reduced intake during the first few months of life. It would follow from this that a dietary deficiency of copper, if it exists at all, must be extremely rare.

Since the plasma copper level in animals is an extremely sensitive indicator of the copper stores, we have measured the plasma copper level in 228 normal individuals (Table IV)

TABLE IV

Normal Plasma Copper

Plasma Cu	No. of patients	% of patients
μg./100 ml.		
90+	218	96
80–90	8	3
68–79	2	1

and in approximately 300 patients with a variety of disorders in an effort to uncover patients with a deficiency of copper.

Studies on patients receiving diets inadequate in at least certain respects, such as patients with scurvy, cirrhosis of the liver, and repatriated American prisoners of World War II, have revealed either a normal or an increased level of copper in the plasma (Table V). Likewise, as shown in Table V, the

plasma copper level has not been found to be reduced in either infants or adults with microcytic hypochromic anemia as a consequence of iron deficiency. Thus, no evidence has been obtained for the existence of even a mild state of copper depletion in human subjects as the consequence of an inadequate dietary intake of copper. Indeed, we have fed diets identical to those which were successful in producing copper deficiency in rapidly growing swine to two infants, five days and eight months of age (Table V). Although this experiment was continued for four and five months, respectively, the plasma copper level remained normal and no anemia developed. This may have been due to the fact that the growth rate of infants is less rapid, or that their copper stores are greater than in young swine; or perhaps the human requirement for copper is less than that of swine. In any event, our experience suggests that copper deficiency as the consequence of an inadequate dietary intake of this element must be very rare in man.

TABLE V

The Plasma Copper Level in Patients with Various Forms of Nutritional Deficiency

Condition	No. of patients	Mean plasma Cu	Range plasma Cu
		μg./100 ml.	*μg./100 ml.*
Normal	228	116	68–161
Cirrhosis	7	136	101–159
Scurvy	2	169	155–183
Malnutrition*	38	148	92–215
Iron deficiency, adults	9	132	102–164
Iron deficiency, infants	26	168	117–225
Milk diet†	2	143‡	109–178
Nutritional hypoalbuminemia	1	155	

* Repatriated American Prisoners of World War II.

† Fed a specially prepared milk diet low in copper for 4 and 5 months, respectively.

‡ At the end of the experimental period.

Although dietary deficiencies of copper may not occur in man, there could be certain conditions (conditioned deficiencies) in human subjects in which copper might not be absorbed or might be excreted in excessively large quantities from the body. With this possibility in mind, we measured the plasma copper level in three patients with sprue (Table V). One of these patients,* who had a severe microcytic hypochromic anemia, was found to have a plasma copper level of 16 μg./100 ml., a red cell copper level of 83 μg./100 ml. of packed cells, a plasma iron level of 12 μg./100 ml., and a total iron-binding capacity of 412 μg./100 ml. Unfortunately, during the baseline period the patient improved markedly on dietotherapy and hospitalization and the copper levels had already begun to increase prior to the administration of copper. Although it seems likely that this patient was depleted of copper as well as iron, this was not proved, because it was not demonstrated that the anemia would not respond to iron alone and required iron-plus-copper therapy.

A second patient with sprue and a long-standing microcytic hypochromic anemia was found to have a plasma copper level of 63 μg./100 ml. and erythrocyte copper concentration of 102 μg./100 ml. of packed cells (normal mean ± 1 S.D., 115 ± 22 μg./100 ml.). A third patient with sprue, but without anemia, was found to have a normal plasma copper level. These preliminary observations indicate an area where further investigations may be worthwhile.

Since approximately 96 per cent of the plasma copper in normal adult subjects is in the form of the blue α-2 globulin, ceruloplasmin, it would seem not unlikely that this copper protein might be excreted in large quantities in the urine of patients with proteinuria. If the loss were great and extended over a prolonged period, it is conceivable that depletion of the body stores might occur. Therefore, to investigate whether a conditioned deficiency of copper takes place in patients with massive proteinuria, the plasma copper, erythrocyte copper, the urine copper, and the hematologic status of a group of patients with the nephrotic syndrome were studied. The results are summarized briefly in Table VI.

The loss of copper in the urine was considerable as compared with the normal and amounted to 0.1 to 0.5 mg./day. Hypocu-

* These studies were carried out in co-operation with Dr. R. E. Hodges, State University of Iowa Medical School, Iowa City.

TABLE VI

Studies on Copper Metabolism in Patients with the Nephrotic Syndrome

Patient	Urine Cu	Urine protein	Plasma Cu	R.B.C. Cu	V.P.R.C.	M.C.V.	M.C.H.C.
	μg./day	*Gm./day*	*μg./100 ml.*	*μg./100 ml.*	*ml./100 ml.*	*cu. μ*	*%*
Normal	9	0.1	116	115	49	87	34
C. E.	270	8.8	63	94	33	82	35
R. K.	490	15.4	58	61	42	88	32
G. H.	346	12.2	94	—	30	80	35
W. B.	183	5.6	89	87	30	88	35
D. G.	324	18.4	49	71	43	89	34
E. C.	92	9.3	20	40	26	88	35

premia was present in four of the six patients. In three of the five patients in whom the amount of copper in the erythrocytes was determined, the value was found to be reduced. In one, the extremely low value of 40 μg. was obtained. Anemia when present was normocytic and normochromic rather than microcytic, and hypochromic. Three patients were treated with copper sulfate orally, and in none was a hemopoietic response observed. Thus, from the limited data available it seems unlikely that the anemia in these patients was related to a deficiency of copper.

Additional studies are needed before the degree of copper depletion in patients with the nephrotic syndrome can be evaluated. The hypocupremia may be due solely to an inability to synthesize ceruloplasmin in the necessary amounts, and the copper stores may not be depleted to any significant degree. On the other hand, the reduction in erythrocyte copper in several of the patients suggests that in at least certain of the tissues of some patients there may be significant depletion of copper. To answer this question, it will be necessary to determine directly the tissue stores of this element.

Hypocupremia has been observed consistently in the newborn and in patients with hepato-lenticular degeneration (Table VII). In both of these conditions it has been adequately demonstrated that the hypocupremia is not associated with a depletion of tissue copper. Indeed, in both conditions tissue copper is markedly increased.

Hypocupremia, a prerequisite for the diagnosis of copper depletion, other than in the conditions noted above, has not been observed in an extensive survey except in one patient with idiopathic hypoproteinemia (Table VII).

TABLE VII

Hypocupremia in Human Subjects

	No. cases	Plasma copper mean	Plasma copper range
		μg./100 ml.	*μg./100 ml.*
Normal	228	116	68–161
Newborn	14	75	45–110
Nephrotic syndrome	16	64	20–96
Hepato-lenticular degeneration	8	51	33–71
Sprue	2	40	16–63
Idiopathic hypoproteinemia	1	60	

From the results of this study, the conclusion is inescapable that the inclusion of copper in the therapeutic armamentarium of the clinician is unnecessary. Further research is required before a decision can be reached concerning the necessity and advisability of copper therapy in some patients with sprue or the nephrotic syndrome. In all other situations studied it would seem that copper therapy is unnecessary, and a wise dictum is that all unnecessary therapy should be avoided.

Cobalt

Physiologic Considerations

The influence of cobalt on erythropoiesis is unique. A deficiency of cobalt results in anemia. The administration of moderate amounts to normal animals produces erythrocytosis, whereas the administration of massive amounts depresses erythropoiesis.

It is now known that cobalt is an essential constituent of the vitamin B_{12} molecule and,

therefore, at least one of its functions in erythropoiesis is concerned with the function of this vitamin.

Cobalt deficiency in ruminants grazing on pastures of low cobalt content has been recognized in many parts of the world, including certain areas in the United States. The deficiency syndrome is characterized by unthriftiness, anorexia, severe anemia, and finally death. The curious observation that cobalt was completely effective when administered orally but totally ineffective when administered intravenously was not understood until recently, when it was demonstrated that the condition is rapidly and completely alleviated by the parenteral administration of about 50 μg. of vitamin B_{12} per day. Apparently, in such animals, vitamin B_{12} is synthesized in the rumen and in the absence of cobalt synthesis cannot take place, so that the animals become deficient in vitamin B_{12}. The fact that vitamin B_{12} is effective in alleviating the syndrome suggests that the only physiologic hemopoietic function which cobalt has is as a constituent of vitamin B_{12}.

It is now well established that the administration of quantities of cobalt far in excess of the normal dietary requirement results in the production of polycythemia in many different species of animals and birds. The polycythemia is a true one and is not due to decrease in plasma volume. It is accompanied by reticulocytosis, hyperplasia of the bone marrow, and increased erythropoietic activity in the spleen and liver. The underlying mechanism responsible for the polycythemia is still obscure. The cobaltous ion is known to have a marked inhibitory action *in vitro* on the endogenous respiration of a number of tissues and is known, under appropriate conditions, to inhibit succinoxidase, choline oxidase, cytochrome oxidase, catalase, choline dehydrogenase, and succinic dehydrogenase. Because of the quantities used to produce polycythemia, it would seem that this is a pharmacological rather than a physiological action of the drug. The suggestion that the effects are due primarily to fixation of thiol (—SH) groups within the tissues is attractive but unproved.

Cobalt is effective in overcoming anemia in animals induced by a low protein diet, hypophysectomy, benzene administration, and inflammation. It should be pointed out, however, that although cobalt alleviates or prevents the development of the anemia associated with inflammation, it does so without benefit to the general nutritional state of the animals. Indeed, in many instances growth of the animals with inflammation is rather poor when cobalt is administered.

Clinical Considerations

Normal adult subjects on self-selected diets consume about 5 to 8 μg. of cobalt per day. Seventy-three to 77 per cent of this amount is absorbed and about 67 per cent of the total daily intake is excreted in the urine. Positive balance is maintained on as little as 5 μg. per day, and the actual requirement is probably less than this amount. In view of this extremely low daily requirement, it is not surprising that a deficiency of cobalt *per se* has never been recognized in a human subject.

In the clinic, cobalt therapy has been used extensively and is gaining wide popularity as a panacea for anemia. There is no doubt that given in sufficient dosage and for a long enough period of time it is effective in alleviating the anemia secondary to infection, cancer, and renal disease. It has been claimed that the rate of hemoglobin regeneration in patients with iron deficiency is more rapid after combined cobalt-iron therapy than with iron therapy alone. The data presented to date are not convincing. The value of this agent in the therapy of chronic hemolytic anemia and aplastic anemia is entirely unconvincing.

Unfortunately, the prolonged administration of cobalt is not without undesirable side effects. Anorexia, nausea, vomiting, skin reactions, severe substernal pain, tinnitus, deafness, paraesthesias of the feet, thyroid hyperplasia with hypothyroidism, and even sudden death* have been observed in patients receiving cobalt therapy. Therefore, the advantages

* Dr. Robert Kark, University of Illinois: Personal communication.

of such therapy must be weighed against the disadvantages. In patients with infection, no evidence has been presented that the patients are benefited by such therapy, even though the mild anemia is corrected. In such patients, it would seem more desirable to correct the underlying cause of the anemia. In some patients with renal disease and severe anemia, cobalt therapy may be of value, but in most patients with iron deficiency anemia, the advantages of a slightly more rapid return of the hemoglobin to normal values would scarcely justify the possible disadvantages of such therapy. In any event, many more carefully controlled studies should be carried out with cobalt before it should be used so widely and indiscriminately. It has come into general use before adequate studies, particularly concerning its toxicity, have been made.

Zinc

Physiologic Considerations

Zinc is known to be an integral component of the enzyme, carbonic anhydrase, and this enzyme is present in erythrocytes and leukocytes. The erythrocytes of normal human subjects contain about 0.0013 μg. of zinc per million cells and the leukocytes contain about 0.032 μg. per million cells.

A severe deficiency of zinc has been difficult to produce in experimental animals. Animals receiving diets low in zinc grow poorly, the fur turns from black to gray, and a mild anemia may develop.

Clinical Considerations

Zinc deficiency has not been observed in humans. The concentration of zinc in erythrocytes of patients with pernicious anemia is elevated, but low zinc concentrations have not been observed consistently in any condition.

Molybdenum

Physiologic Considerations

In cattle and sheep, the excessive ingestion of molybdenum precipitates in most flagrant form the symptoms and manifestations of copper deficiency. In sheep, at least, a paradoxical situation exists, in that the manifestation of copper deficiency co-exists with a concentration of copper in the tissues adequate to maintain normal function. This suggests that molybdenum tends to make copper unavailable for use by the animal, or that it in some way inhibits the copper-containing enzymes.

Clinical Considerations

Claims have appeared that the anemia of iron deficiency responds more rapidly and completely to a combination of iron and molybdenum therapy than to iron therapy alone. Further clinical studies will be necessary before these claims can be accepted as valid.

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For discussion see pages 18 and 19.

DISCUSSION

DR. C. S. DAVIDSON (Boston, Mass.): I'd like to ask Dr. Moore a question.

We have some idea of the iron content of foods in various parts of the world. In Africa, I am told, it is very high; at least the content of the food, as cooked, is pretty high. What about the parts of the world where hypochromic anemia does seem to be related to food? Do we know about the iron content of the food?

DR. C. V. MOORE (St. Louis, Mo.): Some of the data seem to be quite valid. People in the Philippines who have worked on this subject say that the iron intake there is as low as seven or eight milligrams per day, and in adult diets in Britain during the war I believe it was estimated—you probably know this figure better than I—to be about eight milligrams per day. Some of the people working in Central Europe said that during a large portion of the war their values were probably lower than that.

If I may, I'd like to ask Dr. Cartwright a question, Dr. Vilter. The proponents of cobalt therapy have occasionally claimed that cobalt in large amounts—not the physiological but the pharmacological effect of cobalt—is important for the utilization of iron. It seems to me that careful distinction has not always been made between the erythropoietic stimulating effect of cobalt, and increased iron utilization. A statement in Dr. Wintrobe's book interpreted to mean that iron utilization is more efficient when cobalt is given is often cited as partial authority for the opinion. Would Dr. Cartwright tell us how he believes cobalt is related to iron metabolism?

DR. G. E. CARTWRIGHT (Salt Lake City, Utah): We certainly do not believe that cobalt has any specific effect on iron metabolism. During the period that polycythemia develops, when one gives cobalt to an animal, there is, of course, an increased uptake of iron in absolute number of milligrams; also one can show that there is more iron absorbed from the gastrointestinal tract during that period. But those are effects that one would see with rapid regeneration of blood under any circumstances, and certainly there is no known specific action of cobalt on iron metabolism.

DR. M. A. BLANKENHORN (Cincinnati, Ohio): Mr. Chairman, I'd like to ask Dr. Moore, in his sweating experiments, if that was good, honest sweat associated with vigorous physical activity? Because, Dr. Moore, in all of your balance studies on iron, you did not mention the work factor, nor in Dr. Wintrobe's book, as to the trauma of circulation.

DR. MOORE: Our sweat was not good, honest sweat. It was collected by having subjects wear a plastic suit and stay in a hot room. In two hours time we could collect as much as a liter of sweat. It was a very debilitating experience. Several of our subjects became faint during that period of time. The conditions were artificial.

The real reason that we did it, however, was because of the claim that approximately six to nine milligrams of iron were lost in sweat by a normal adult male in a day's time. That figure is so high that it would be virtually impossible for us to assimilate enough food iron to compensate for the loss, and all of us should have hypochromic anemia. Our figures were really calculated in such a way as to give what we thought would be maximum values that could be lost.

You may criticize these results because we didn't take into consideration the work factor; perhaps we should have had a bicycle to provide work for the subjects, but that was not done.

DR. C. D. MAY (Iowa City, Iowa): I'd like to ask Dr. Moore, if one carried on a continuous type of study (if he had an opportunity to do so) whether, after the initial improvement in absorption of iron by the supplementation of the large amount of ascorbic acid, that can be repeated day after day so that this improvement in absorption is a regular thing, or will it not reflect the saturation of the subject with ascorbic acid?

DR. R. W. VILTER (Cincinnati, Ohio): Dr. May wants to know whether continuous feeding of ascorbic acid will continue the increment of iron absorption that Dr. Moore reported for his single day experiments.

DR. MOORE: The answer is no; we have done only acute experiments. Ascorbic acid was given parenterally, however, in rather large amounts without effect on iron absorption.

DR. B. CONNOR JOHNSON (Urbana, Ill): In regard to Dr. Cartwright's comments, as far as I know there is no evidence for a nutritional requirement for cobalt.

I think it is well established from the Lederle work and others, and the work at Wisconsin, that there is a definite function of molybdenum for specific enzymes. I think we should consider molybdenum essential even though toxic at higher levels.

I'd also like to address a question to Dr. Moore about the Illinois work he referred to regarding iron excretion in the sweat, as to what explanation he might have for the differences in iron excretion. In the Illinois work the subjects were kept at high temperature, and high humidities for eight hours a day over considerable periods of time, and we did have excreted as much as ten liters of what one might call normal sweat in a one-day period.

DR. CARTWRIGHT: In regard to the comments about molybdenum, they were in relationship to hematopoiesis.

I did not mean to imply that the daily dietary requirement for cobalt is 5 micrograms. It is no more than this, and there may be no requirement at all except for that which is in the vitamin B_{12} molecule.

DR. VILTER: Dr. Moore, do you want to speak on the sweating problem again?

DR. MOORE: I can't explain the high iron content of the sweat in the Illinois experiments. Iron is so omnipresent, however, that it would be difficult to avoid contamination even with the elaborate precautions taken. One avoids that danger by using isotopic iron.

Some Metabolic Interrelationships of Folic Acid, Vitamin B_{12}, *and* Ascorbic Acid

By J. N. WILLIAMS, JR., PH.D.*

IN THE field of vitamin research it is doubtful if there has been more apparently contradictory published research than that concerning the roles of folic acid and vitamin B_{12} in metabolism. The reason for this state of affairs is not clear, unless it can be attributed to slight variations in experimental approach from one laboratory to another. Minor changes in apparently unimportant nutrients, differences in management of animals, differences in types of the organisms, tissue or organ studied: all of these could account to some extent for the differences of opinion concerning the mechanism of action of folic acid and particularly of vitamin B_{12}. In this discussion I shall try to make as clear a picture as possible of the most generally accepted concepts sifted from the vast amount of literature on this subject.

When one speaks of the functions of folic acid, it is very difficult at many points to omit reference to vitamin B_{12}. In several instances these two vitamins are apparently involved in entirely separate functions. In many other instances, however, one of these factors cannot be considered without bringing the other into the discussion. The underlying reason for this has not been discovered. In the title of this paper, ascorbic acid is included in addition to folic acid and vitamin B_{12}. We shall see later in this discussion how ascorbic acid may be related to the metabolism of these factors.

As it happens in most cases, study of a particular biological factor begins with the observation that the lack of this factor produces a noticeable change in some characteristic of a certain living organism. Then begins the sometimes long and tedious process of isolation, characterization, and study of how the factor functions in the vital process. In the case of folic acid, vitamin B_{12}, and ascorbic acid, the isolation process has been completed. Except for vitamin B_{12}, the process of chemical characterization has been mainly completed. I say mainly because there are many indications that the end of this process for folic acid has not yet been reached. The many different derivatives of folic acid are still being studied, and from all indications there are more natural forms of these substances than had previously been supposed.[1,2] The way in which folic acid (and its derivatives), vitamin B_{12}, and ascorbic acid actually function in the vital process is the topic of the present discussion. Unfortunately, this is the portion of the research on these factors which is the least clear and which in many cases is downright muddled.

Perhaps the most significant discovery which opened up the field for investigations on the metabolic role of folic acid was the discovery of the *Leuconostoc citrovorum*-8081 factor[3] variously called *citrovorum* factor, folinic acid-SF, folinic acid, LCF, CF, leucovorin, and N^5-formyl derivative of 5,6,7,8-tetrahydrofolic acid. (From the recent discovery that *Leuconostoc citrovorum* is not an authentic *Leuconostoc citrovorum* but is actually a typical strain of *Pediococcus cerevisiae*,[4] the significance of some of the names of folinic acid is decreased. For example, this factor might better be called *Pediococcus cerevisiae* factor or PCF.) (Fig. 1.) From the figure, which includes for the sake of comparison both folic acid and folinic acid, one can see that significant differences

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* Associate Professor of Biochemistry.

between the two factors exist: (1) Reduction of one of the rings of folic acid and (2) introduction of a formyl group at position 5. As

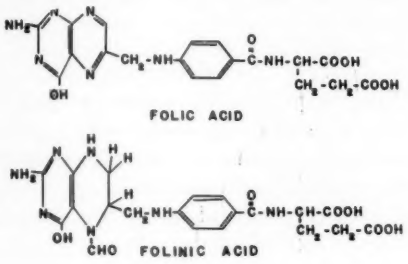

Fig. 1. Structural formula of folic and folinic acids.

we shall see later, the presence of the formyl group has given quite an impetus to the imagination of investigators in this field as to the possible functions of folinic acid. Leucovorin, a synthetic form of folinic acid, has been found to possess about one-half the biological activity of the natural factor. This has been explained quite satisfactorily as being due to the production of an asymmetric center at carbon atom 6, so that 2 isomers are formed, only one of which is biologically active.

Because of the tendency among biochemists to think of the functions of vitamins in terms of coenzymes, the question necessarily arises as to what is the actual coenzyme derived from folic acid. It is possible that folinic acid itself is one coenzyme derivable from folic acid. It has been conjectured, however, that folinic acid is only an intermediate in the formation of a true coenzyme from this vitamin. It cannot be stated at present which is the correct interpretation. In certain *in vitro* enzyme systems, however, folinic acid definitely stimulates reaction and has been called formylated Coenzyme F.[5]

Although the isolation of vitamin B_{12} was made a number of years ago, its chemical characterization is by no means complete. For that reason its structural formula cannot be offered. Perhaps because of the lack of knowledge concerning the complete structure of vitamin B_{12}, knowledge of the actual function of this vitamin in metabolism has not advanced very far. If the chemical structure were known, possibilities concerning its specific function could be surmised much more easily. By indirect reasoning, however, a fair amount of knowledge has been obtained on the function of this vitamin.

Ascorbic acid has been included in this discussion because of some of its observed relationships with folic acid in scorbutic animals.[6–13] It has also been found that ascorbic acid augments the conversion of folic acid to folinic acid in liver and kidney systems *in vitro*.[14] Other workers have shown that the simultaneous administration of folic acid and ascorbic acid in human beings gives a urinary excretion of folinic acid which is about three times greater than if folic acid is administered alone.[10] Therefore, it appears that, in addition to its other many functions in metabolism, ascorbic acid is also involved in the utilization and indirectly in the functions of another vitamin, folic acid.

With this brief introduction let us turn next to a discussion of some of the more specific relationships of these vitamins to metabolic reactions. The idea that folinic acid might be related to the transfer of a single-carbon fragment has gained ground in recent years and is generally accepted by many workers in this field. The presence of the formyl group in folinic acid adds considerable weight to this concept. A large number of different metabolic reactions can be imagined to occur *via* the transfer (withdrawal or introduction) of a one-carbon fragment. In the present discussion it should be emphasized, however, that wherever the transfer of a one-carbon fragment is mentioned, this does not necessarily mean that only a single reaction is involved. The seemingly simple reactions may involve none, one, or more reactions and co-factors in addition to a co-factor derived from folic acid.

Some of the substances whose metabolism has been related to folic acid and vitamin B_{12} are the following: thymine and its desoxyriboside, thymidine; the purines; glycine and serine; methionine, choline, and other compounds with labile methyl groups; histidine; tryptophane; and heme. Other substances could be included here, but these are perhaps the best established at the present time.

Thymine and Thymidine

In 1941 it was observed that a combination of thymine and purines could replace folic acid (which was uncharacterized at that time) in the nutrition of certain micro-organisms.[15,16] Since then, a vast amount of research has been carried out on this relationship; and with apologies to the people who did the excellent work in this field, only a few individual reports can be discussed here. It has been shown that large oral doses of thymine give definite hematologic responses in pernicious anemia. Evidence also exists that large doses of thymine produce hematologic responses in folic acid-deficient swine.[17] Smaller doses of thymidine induce responses in pernicious anemia cases in relapse.[18] How and why these results should be obtained is a difficult question to answer, especially in view of the fact that exogenous thymine does not appear to be utilized to any great extent by animals, as shown by isotope studies.[19] It is possible that, if the endogenous production of thymine or thymidine is blocked by a folic acid or vitamin B_{12} deficiency, huge doses of the pyrimidine or its desoxynucleoside might be utilized by mass action to an extent great enough to produce some response. The utilization of one-carbon donors to form the methyl group of thymine (Fig. 2) has

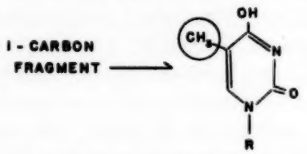

Fig. 2. Production of the methyl group of thymine from 1-carbon fragments.

been demonstrated using isotopically labeled compounds.[20,21] With the hindsight that folinic acid may be involved in one-carbon transport, because of its chemical structure, it is possible to visualize that this factor could be involved in the endogenous synthesis of thymine. The large number of experiments carried out with micro-organisms in the study of folic acid, vitamin B_{12}, thymine, and thymidine interrelationships, too numerous to discuss here, have led some workers to the conclusion that folic acid is probably more closely involved in the production of thymine and that vitamin B_{12} is more related to the production of the desoxyribose portion of thymidine.

Purines

With the purines the picture is somewhat clearer than with thymine and thymidine, although the mechanisms of vitamin involvement are still hypothetical to a great extent. The basic structure of the purine pentosides and pentotides is shown in Figure 3.

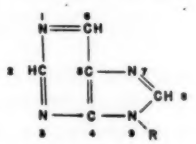

Fig. 3. Purine skeletal structure.

In 1950, Skipper, Mitchell, and Bennett[22] observed that an antagonist-induced deficiency of folic acid in mice caused a decreased incorporation of C^{14}-labeled formate into nucleic acid purines. In 1951, these studies were extended to rats by Drysdale, Plaut, and Lardy,[23] who found that rats with a folic acid deficiency incorporated less labeled formate into carbon atoms 2 and 8 of liver purines than did rats receiving folic acid. Through the studies of Barnes and Schoenheimer, Buchanan and co-workers, and Shemin and Rittenberg,[24–29] the precursors of the purine skeleton have been pretty well established (Fig. 4).

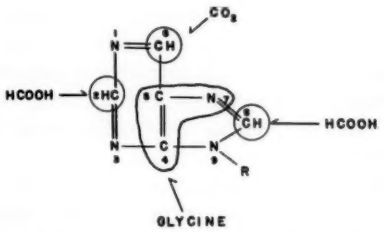

Fig. 4. Probable precursors of the carbons of purines.

Atoms 4, 5, and 7 arise from glycine; 6, from carbon dioxide; and 2 and 8 from formate. The remaining nitrogen atoms (1, 3, and 9) apparently do not arise from ammonia directly, but from the amide nitrogen of glutamine and the amino nitrogen of glutamic

acid. Our main interest in this discussion surrounds the introduction of carbon atoms 2 and 8 into the purine nucleus, since much experimental evidence indicates that folinic acid is in some way involved with these two carbon atoms. In the figure, an R group is attached to nitrogen atom 9. This group can be either ribose or desoxyribose, depending upon the type of purine pentoside or pentotide. From the work of Greenberg,[30] it appears at least in the case of hypoxanthine, that the purine ring is completed only after introduction of ribose phosphate.

Using bacteria, Stetten and Fox[31] and Shive and co-workers[32] have shown that, if sulfonamides are included in the growth medium, an intermediate, 5-amino-4-imidazolecarboxamide (Fig. 5) accumulates in the medium.

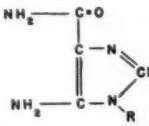

Fig. 5. 5-amino-4-imidazolecarboxamide.

The relationship of this substance to the purines is quite evident. If a carbon atom (carbon atom 2 of the purine nucleus) is introduced connecting the free amino groups, a purine is obtained. In *in vitro* work using pigeon liver extracts, Buchanan and co-workers[5] found that, if folic acid was added to the incubation mixture in which the incorporation of C^{14}-labeled formate into inosinic acid was studied, only slight stimulation was obtained. However, when folinic acid was added to the mixture, a marked stimulation of formate incorporation into carbon 2 of inosinic acid was obtained. There was some evidence that the incorporation of glycine and carbon 8 from formate was increased by the addition of folinic acid, even though the system was not set up to study these reactions directly. In experiments by Berg[33] and Greenberg,[34] evidence has been obtained that homocysteine in addition to folinic acid may be involved as a one-carbon fragment carrier. It was found that the addition of homocysteine to a briefly dialyzed pig liver preparation (to remove substrate preferentially to co-factors) increased the incorporation of formate into carbons 2 and 8 of inosinic acid. Exactly what the relationship of folinic acid and homocysteine is to each other and to the actual mechanism by which formate is incorporated into the purine nucleus remains to be investigated further.

Glycine and Serine

A great deal of confusion exists concerning the relationship of folic acid and vitamin B_{12} to glycine, serine, and formate metabolism. Many contradictory results have been reported, which can probably be traced to differences in the dietary regimens fed experimental animals, as well as to other more nebulous variables. Fairly early in the study of the possible roles of folic acid and vitamin B_{12} in metabolism, it was observed that the toxicity of high levels of glycine in the diet could be reversed both by folic acid[35,36] and vitamin B_{12}.[37–39] With this demonstration of a relationship between these vitamins and glycine, further studies were in order. In 1950, Plaut, Betheil, and Lardy[40] observed that rats deficient in folic acid incorporated less C^{14}-labeled formate into certain amino acids, especially serine, than did rats fed folic acid. In later studies, Stekol and co-workers[41,42] and Arnstein and Neuberger[43,44] found that vitamin B_{12} may be involved in the utilization of glycine to form serine. It had earlier been shown by Sakami,[45] using labeled formate, that glycine plus formate is very efficiently converted to serine. Also it is known that the α-carbon of glycine can give rise to a one-carbon fragment and that the β-carbon of serine can do the same. Sakami and Welch[46] found that labile methyl groups can be formed from formate. Thus, because of the apparent interconvertibility of these substances, labile methyl groups can also arise from the α-carbon of glycine and the β-carbon of serine. These reactions are summarized in Figure 6.

A study of the effects of folic acid and vitamin B_{12} on any part of this picture is complicated by the fact that many different enzyme systems are involved. Thus, the relative *rates* of conversion of one substance to another *in vivo* are concomitantly compli-

cated by this fact, and results may be easily misinterpreted. For example, the degree of relative labeling of the ethanolamine portion and the methyl groups of choline could be obscured by differences in the rates of enzymatic formation of ethanolamine and methyl groups from, say, glycine. This situation could also account in part for some of the contradictory results concerning the effects of a vitamin B_{12} deficiency on the synthesis of labile methyl groups from formate, for example. In Figure 6, formate has been placed in quotes because of the observation by Elwyn and co-workers[47] that deuterium-labeled serine was converted (*via* its β-hydroxy methyl group) to choline methyl groups without loss of the two hydrogen atoms on the β-carbon. These results indicate that the hydroxymethyl group of serine is not oxidized to the level of formate before transfer. Therefore, some doubt exists concerning the role of folinic acid in the transfer of this group from serine, because the formyl group of folinic acid, *as such*, could not take part in this transfer. It is possible, however, as many workers have surmised, that a hydroxymethyl form of folinic acid exists, in which case the factor could enter into the transfer mechanism.

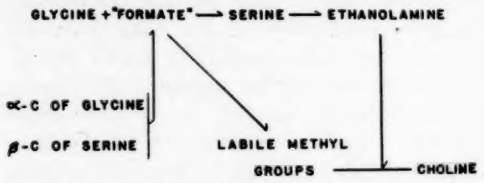

Fig. 6. Summary of glycine-serine-choline interrelationships.

Methionine, Choline, and Betaine

In the following section, studies on the effect of folic acid and vitamin B_{12} on the synthesis of methionine and choline methyl groups will be considered in more detail. Also, other factors, such as transmethylation and conversion of choline to betaine will be considered. The reactions are briefly outlined in Figure 7.

Sakami and Welch,[46] in a preliminary report, indicated that liver slices from folic acid-deficient rats converted less formate to methionine methyl than did slices from folic acid-supplemented animals. A similar result was obtained by Stekol and co-workers[41] in *in vivo* experiments. The picture concerning the relationship of vitamin B_{12} to methyl synthesis from formate is somewhat confused. Some workers have indicated that vitamin B_{12} is involved,[41,42] while others have obtained negative results.[43,44] For this reason the correct interpretation as far as vitamin B_{12} is concerned cannot be made with certainty.

In the scheme presented in Figure 7, methionine methyl is indicated as being a precursor of choline methyl by a reaction labeled Transmethylation I. Recently Cantoni[48,49] has shown that, in the transfer of a methyl group from methionine, the formation of an intermediate "activated methionine" is necessary. Since this reaction, which involves a chemical combination of ATP and methionine, and the transmethylation resulting from this intermediate are apparently not related to folic acid or vitamin B_{12}, we will limit our discussion to Transmethylation II. This second type of transmethylation concerns the transfer of a methyl group from betaine to homocysteine to form methionine. This reaction is associated with folic acid and particularly with vitamin B_{12}. Nutritional experiments by a number of workers have been interpreted as implicating vitamin B_{12} in the transfer of a methyl group of betaine to homocysteine.[50–56] Again, however, we are faced with contradictory results by other workers, which indicate that vitamin B_{12} is associated only with *de novo* synthesis of methyl groups and not with the transmethylation reac-

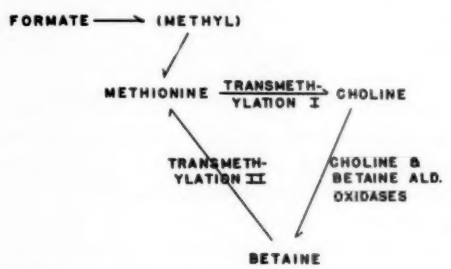

Fig. 7. Summary of the transmethylation cycle.

tion.[57-60] At present these discrepancies cannot be explained except on the basis of differences in diet and possibly by the degree of vitamin B_{12} deficiency.

In 1950, Oginsky[61] observed that liver homogenates from rats deficient in vitamin B_{12} demonstrated a reduced ability to methylate homocysteine using betaine as a methyl donor. These studies were repeated by other workers[62] who arrived at the same conclusions. The latter work was extended,[63] and the effects of a single injection of vitamin B_{12} on the reactivation or repletion of the betaine-homocysteine transmethylase system were studied. It was found that this transmethylase regained its normal activity within one day (which was the earliest time measured) after the vitamin B_{12} injection. Although none of these experiments represent proof that the vitamin is a direct activator of betaine-homocysteine transmethylase, at least they indicate that it is quite closely associated with maintenance of activity of the enzyme. It is also possible that if vitamin B_{12} is not actually a part of the co-factor for the enzyme, it may be involved either in the genesis of the co-factor or in the activation of certain groups of the apoenzyme. These questions cannot be answered with certainty until the enzyme is isolated in a relatively pure form. A report has been made quite recently[64] that betaine-homocysteine transmethylase has been partially purified (about sixty-fold), that it has been separated into a heat-stable co-factor and a heat-labile apoenzyme, but that a typical vitamin B_{12} spectrum was not exhibited either for the holoenzyme or for the component parts. Mistry *et al.* (*Fed. Proc.* 13: 265, 1954 and Firth *et al.* (*Proc. Soc. Exp. Biol. & Med.* 85: 307, 1954) have reported that in vitamin B_{12}-deficient pigs the transfer of methyl groups from methionine to guanidoacetic acid to form creatine, from betaine to homocysteine to form methionine, or from methionine to form choline is unimpaired. However, in work with rats, Johnson's group did find a depression in liver betaine-homocysteine transmethylase when the animals were made deficient in vitamin B_{12} (personal communication).

In the outline shown in Figure 7, choline must be converted to betaine before methyl group transfer to homocysteine can occur.[65,66] A number of workers[67-72] have studied the effects of a folic acid deficiency on the enzyme system, choline oxidase, which is involved in the conversion of choline to betaine. The most dramatic effects have been obtained when aminopterin is administered to animals. In these studies, choline oxidase activity is depressed quite markedly by aminopterin. It has been difficult to demonstrate a similar effect with a simple folic acid deficiency. The latter phase of these studies was investigated in detail,[73] and it was found that a depression of liver mitochondrial choline oxidase by a simple folic acid deficiency could be demonstrated by adjusting the concentration of choline *in vitro* to the optimum concentration for the enzyme. Assay of the mitochondrial preparations from control and folic acid-deficient animals for folinic acid indicated a fair correlation between choline oxidase activity and folinic acid activity. In quite recent work Dinning and co-workers,[72] using folic acid-deficient chicks, have observed a complete loss of choline oxidase activity of chick bone marrow cells. Whether folic acid is directly or indirectly involved as a co-factor for choline oxidase cannot yet be answered. However, if there is loss of activity of this enzyme, whether by loss of co-factor or for some other reason, the resulting detrimental effects on one-carbon metabolism in animals become obvious because of the position of the enzyme system in the transmethylation cycle. This could be particularly harmful in bone marrow cells where a simple folic acid deficiency causes complete disappearance of the enzyme.

Histidine

Turning now to the possible involvement of folic acid in the metabolism of another amino acid, histidine, Bakerman and co-workers[74] observed that rats fed succinylsulfathiazole without folic acid excreted five to ten times as much glutamic acid as their pair-fed controls. The glutamic acid was present as a labile precursor which could be converted to

the amino acid by chemical means.[75] The enzymatic breakdown of histidine has been shown to yield ammonia and an intermediate product which could be broken down further to glutamic acid, formate, and ammonia.[76,77] Thus histidine has been associated indirectly with folic acid through the glutamic acid excretion studies and *via* its ability to give rise to formate.

Tryptophane

If one considers the unique metabolism of tryptophane in its conversion to the pyridino structure, an intermediate called formylkynurenine[78,79] (Fig. 8) is observed. The conversion of this intermediate to kynurenine, as shown in the figure, must involve the removal of the formyl group. No direct evidence for the association of folic acid with this conversion has been demonstrated although excretion studies[80] have indicated that folic acid-deficient, tryptophane-supplemented rats excrete considerably less niacin or N-methylnicotinamide than controls. In some recent studies conducted by us,[81] this problem has been investigated further by studying the ability of folic acid-deficient rats to form blood pyridine nucleotides and N-methylnicotinamide from tryptophane and from niacin. In these studies it was observed that aminopterin-treated rats were totally unable to form the pyridine nucleotides and N-methylnicotinamide from tryptophane, but they were able to produce them practically unimpaired from niacin. These results indicate that folic acid is necessary for the conversion of tryptophane to pyridine compounds. Exactly at what point folic acid is involved must be solved by future experimentation. However, in view of the known association of folic acid with one-carbon metabolism, it is possible that the vitamin is involved in the conversion of formylkynurenine to kynurenine.

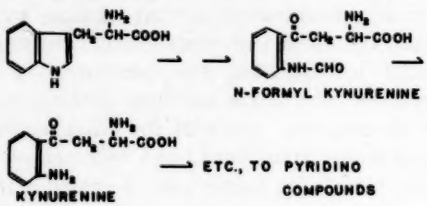

Fig. 8. Brief summary of the conversion of tryptophane to pyridino compounds.

Protoporphyrin

A substance of great importance to students of blood metabolism is heme. As an example of the last type of compound which will be discussed and with which folic acid and/or vitamin B_{12} may be connected, I have chosen protoporphyrin, the porphin of heme. In Figure 9 is presented the structure of this compound. You will notice that certain carbon and nitrogen atoms have been encircled. Wittenberg and Shemin[82] have shown that the encircled carbon atoms arise from the α-carbon of glycine. It has also been found that the nitrogen atoms of the pyrrole rings arise from glycine.[83–88] Since the α-carbon of glycine is utilized for porphyrin synthesis, it is not difficult to imagine that folic acid and vitamin B_{12} may be closely related to synthesis of this compound because of the interrelationships discussed earlier under *Glycine and Serine*. Indeed, Plaut, Betheil, and Lardy[40] found that folic acid-deficient rats incorporated less labeled formate into heme than did pair-fed control rats. While this is not unequivocal proof that folic acid is directly involved in porphyrin synthesis, it is at least indirect evidence that this may be the case.

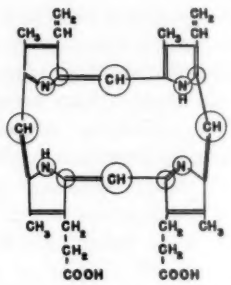

Fig. 9. Protoporphyrin, the porphin of heme.

Conclusion

From the wealth of reports in the literature concerning the possible functions of folic acid and vitamin B_{12} in metabolism, we are still

quite some distance from assigning definite roles to each of these vitamins. From knowledge of the chemical structure of folinic acid, a boon was given to the development of ideas concerning its role in one-carbon transfer. Our lack of similar ideas for vitamin B_{12} can possibly be accounted for by our lack of knowledge of its complete chemical structure. There are many blank spots in the picture explaining why these two vitamins sometimes apparently overlap at many points in their metabolic functions. The precise relationship between folic acid and vitamin B_{12} remains to be determined. Many of the reactions they apparently catalyze are based on evidence that must be listed as indirect and circumstantial. In the case of these vitamins, as it has so often happened with other vitamins, general effects of vitamin deficiencies have led to more specific studies dealing with more and more simplified systems. Eventually it is hoped that individual, single-step reactions can be studied in purified, isolated systems, at which time we will be closer to understanding the fundamental mechanism of action of these vitamins in metabolism.

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Interrelationship of Folic Acid, Vitamin B_{12} *and* Ascorbic Acid *in Patients with* Megaloblastic Anemia

By JOHN F. MUELLER, M.D.* AND JOHN J. WILL, M.D.*

In 1926 Minot[1] noted hematologic improvement in patients with pernicious anemia who were fed large amounts of liver. Later he demonstrated that crude liver extracts, given parenterally, induced similar responses. These observations began two and one-half decades of investigation to determine the mechanisms involved. It was soon found that the liver factor was present in extremely small amounts in the crude preparations. Refining processes were employed which eventually culminated in the isolation and crystallization of vitamin B_{12}. The possibility arose that at least one other factor was essential for normal maturation when, in 1939, Wintrobe[2] demonstrated that yeast, administered orally, had a hematologic effect in pernicious anemia. Castle[3] had shown in 1928 that the defect in pernicious anemia was the lack of a substance normally present in gastric juice and called intrinsic factor, without which extrinsic or food factor (vitamin B_{12}) is poorly absorbed. Vitamin B_{12} by the oral route, therefore, is not effective unless it is given in massive amounts, and the response from yeast must have been due to some other substance. Furthermore, Lucy Wills[4] and others had studied certain patients with megaloblastic anemias who were refractory to refined liver extract given parenterally but did respond to oral therapy with crude liver or yeast preparations. Thus, it was not surprising when folic acid was isolated from liver and other foodstuff and shown to be effective orally in megaloblastic anemias. However, the remission induced in pernicious anemia by folic acid proved to be temporary, and neurologic deterioration often progressed.

That ascorbic acid was in some way concerned with this problem was indicated by the observation that vitamin C induced reticulocyte responses and minor red cell rises in many patients with pernicious anemia and that pernicious anemia patients on low ascorbic acid intakes required more liver extract or vitamin B_{12} to maintain satisfactory remissions than those with normal vitamin C intakes.

CONCEPTS OF NUTRITIONAL INTERRELATIONS

It is evident that these materials are hematologically effective, but the mechanisms by which they produce remissions or the relationships of one to the other are not so clear. By 1948 our laboratory had had considerable experience with the remissions and relapses which occurred in patients with pernicious anemia on folic acid, and, in addition, we had carried out some studies on a patient with refractory megaloblastic anemia. We knew also at this time that thymine would replace folic acid in metabolic processes essential for the growth of *L. Casei*[5] and that thymidine would replace vitamin B_{12} in a similar fashion for *L. Leishmannii*,[6] but that the effective level for the pyrimidine compounds was 1000 to 1500 times that of the vitamins. With these data in mind, we proposed a formula to explain the chemical interrelationships of vitamin B_{12} and folic acid in bringing about the maturation of the pernicious anemia megaloblast (Figure 1).

This scheme, in retrospect much too simple, suggested that folic acid and vitamin B_{12} function as catalysts in a chemical chain reaction culminating in the normal production of

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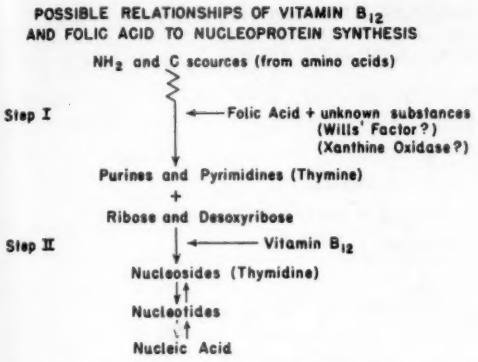

Fig. 1. A simplified scheme of the earlier concepts of the action of folic acid and vitamin B_{12} (Vilter et al. *Blood* 5: 695, 1950).

nucleoproteins, and that a deficiency of vitamin B_{12} was the basic abnormality in pernicious anemia. Folic acid was only secondarily involved, and produced a remission in pernicious anemia by a mass action effect in pushing the reaction to completion.

However, as more information became available, the simple scheme had to undergo addition and revision, as Figure 2 indicates. In this diagram the metabolism of folic acid is depicted and the proposed relationship of ascorbic acid to this reaction is shown. Also depicted are the various megaloblastic anemias and the abnormalities in this reaction which are probably responsible for their occurrence.

Further data, among which were the experiments which clearly indicate the effect of vitamin B_{12} on folic acid metabolism, necessitated an even more complex scheme (Figure 3).

This concept, derived from the work of many investigators, still has as its basic structure that proposed before, namely, that vitamin B_{12} and folic acid act as catalysts in a reaction which leads to the formation of nucleic acid, and that ascorbic acid has an indirect effect on folic acid metabolism in at least two different sites. To this must be added the assumption that vitamin B_{12} in some way is concerned with folic acid metabolism and a deficiency of the former (vitamin B_{12}) will result in a conditioned deficiency of the latter (folic acid).

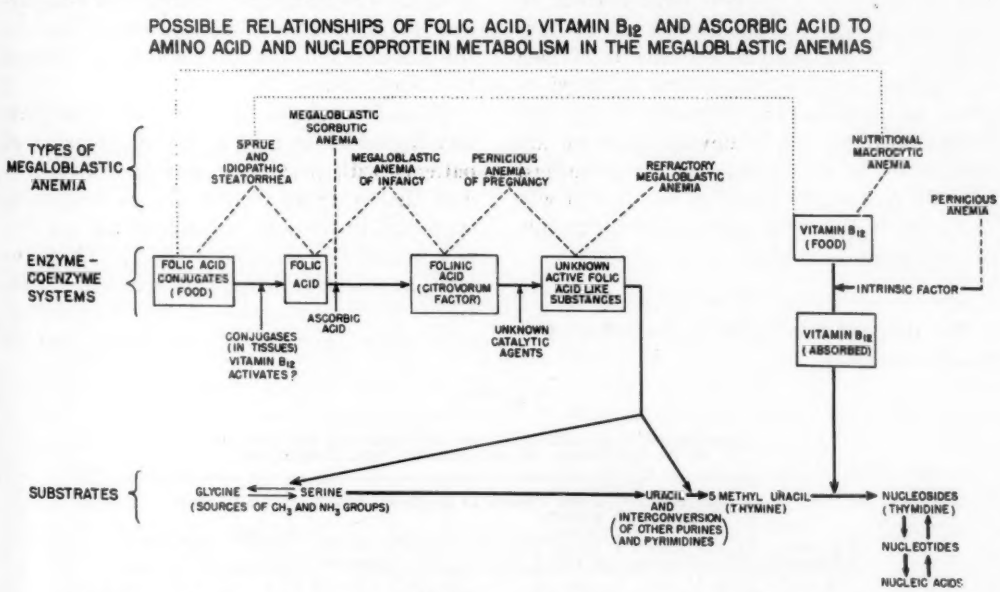

Fig. 2. A revised scheme of the possible interrelationships of nutrient factors in erythropoiesis.

In this symposium Dr. Williams has discussed many of the chemical, microbiological, and animal experiments which have contributed so much to an understanding of this problem. The report we wish to make concerns

SCHEMATIC REPRESENTATION OF THE POSSIBLE INTERRELATIONSHIPS OF VITAMIN B_{12}, FOLIC ACID AND ASCORBIC ACID IN NUCLEIC ACID METABOLISM OF PATIENTS WITH MEGALOBLASTIC ANEMIA

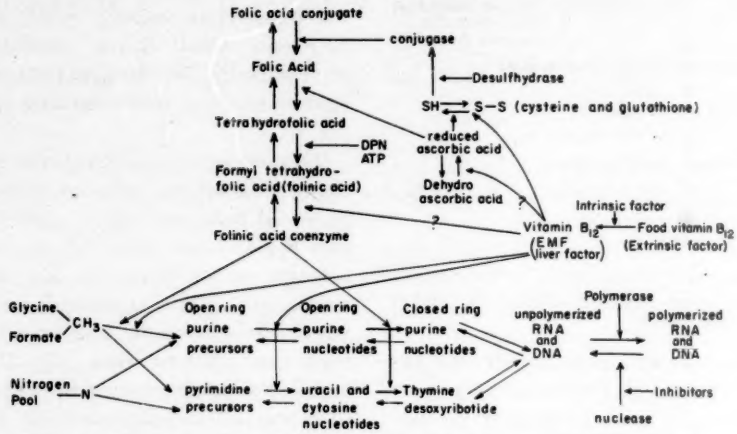

Fig. 3. Present concept of nutrient metabolism in megaloblastic anemias.

some of the clinical experiments our laboratory has performed over the past eight years in an attempt to test various aspects of the hypothesis. These experiments can be divided into groups, each of which was designed to prove or disprove one segment of the hypothesis. It should be obvious that we are unable to discuss all experiments performed over the past eight years, but an attempt will be made to discuss a representative example of each group of studies.

ACTION OF FOLIC ACID

The propositions which we wished to test are the following:

1. That folic acid can produce temporary remissions in pernicious anemia by a mass action effect, and in so doing further depletes the already deficient body stores of vitamin B_{12} (Table I).

Table I compares the effectiveness of vitamin B_{12} and folic acid in the maintenance of patients with pernicious anemia. It is evident that whereas vitamin B_{12} is completely successful in doses of one microgram per day or less, folic acid is undependable, and hematologic, as well as neurologic, relapse is frequent. However, patients who relapse hematologically while taking folic acid often may be

TABLE I

A Comparison of the Effectiveness of Vitamin B_{12} and Folic Acid in the Maintenance of Patients with Pernicious Anemia

# of Yrs. maintained	Vitamin B_{12} 10 gamma I.M. every 2 weeks or 20 gamma I.M. monthly 44 Patients				Folic Acid 30 mg. orally 3 times a week 36 Patients			
	# of Pts.	Hemat. Relapse	Neuro. Relapse	Died or Lost	# of Pts.	Hemat. Relapse	Neuro Relapse	Died or Lost
1	19	0	0	7	36	1	5	1
2	12	0	0	0	31	3	2	2
3	7	0	0	0	23	11	9	1
4	4	0	0	0	11	1	1	0
5	2	0	0	0	10	0	0	3
6					7	1	1	1
7					5	0	0	0

controlled temporarily by increasing the amount of folic acid given.

Figure 4 demonstrates the rather marked hematologic relapse which occurred in a patient with pernicious anemia on 30 mg. of folic acid three times a week. A partial remission resulted from increasing the dosage to 50 mg. per day. The moderate improvement in the patient's neurologic condition was attributed primarily to an increase in the amount of hemoglobin plus better nutrition. This remission was short-lived, however, and relapse occurred in two and one-half months, necessitating therapy with refined liver extract.

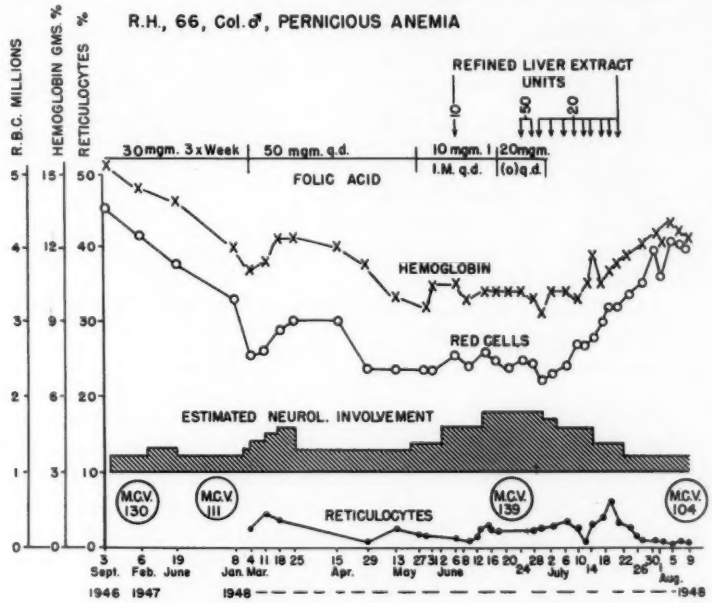

Fig. 4. Folic acid relapse in a case of pernicious anemia treated with large doses of folic acid and refined liver extract (Vilter et al. *Blood* 5: 695, 1950).

It should be noted that the relapse on folic acid is associated with a hypocellular, nonmegaloblastic bone marrow and the response to vitamin B_{12} is a slow one, unassociated with a reticulocyte response.

We interpret these observations as supporting the hypothesis that folic acid must work by mass action effect, pushing the reaction in Figure 3 to completion, thereby further depleting vitamin B_{12} and aggravating the neurologic disorder which must be produced primarily by vitamin B_{12} deficiency. This severe deficiency is reflected by an acellular, nonmegaloblastic bone marrow which responds only slowly to replacement therapy with vitamin B_{12}. This situation is probably similar to the accelularity which develops as a result of severe aminopterin toxicity. In this instance, folic acid activity is so severely inhibited by the antagonist that nucleic acid production is nearly stopped.

ROLE OF VITAMIN B_{12}

2. A second proposition is: a deficiency of vitamin B_{12} in some way induces folic acid deficiency, since a dietary deficiency of folic acid does not exist in the patient with pernicious anemia; yet such a patient does respond hematologically to treatment with folic acid.

In 1947 Bethell and his group[7] showed that patients with pernicious anemia given the hexaglutamyl conjugate of folic acid orally did not excrete as much folic acid in the urine before the administration of vitamin B_{12} as after it. Nor did the conjugate have as much hematologic effect as folic acid. This sug-

gested that vitamin B_{12} was necessary for the action of conjugase which liberated folic acid from the conjugated form in which it is present in food. That this is not always true is evident from the fact that normal blood values for folic acid were found in 9 out of 16 cases of pernicious anemia in relapse by Nieweg *et al.*[8] Therefore, it has been suggested that vitamin B_{12} may have a role elsewhere in the folic acid cycle.

It has been suggested by Girdwood,[9] Witts,[10] and others that vitamin B_{12} has something to do with the mobilization and conversion of folic acid to folinic acid, although Jukes[11] was unable to show this in the rat. If this were the case, we would expect that folinic acid, milligram for milligram, would be more effective than folic acid in pernicious anemia in relapse. This does not appear to be the case.

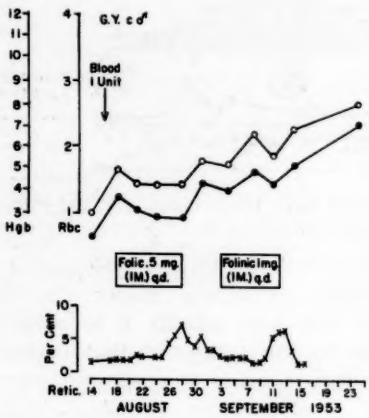

Fig. 5. Comparison of suboptimal doses of folic and folinic acids in a case of pernicious anemia in relapse.

It is evident from Figure 5 that there is no significant difference between comparable doses of folic and folinic acids, regardless of which is given first (see Figure 6).

If vitamin B_{12} had a role in folic acid metabolism, one might expect suboptimal doses of vitamin B_{12} plus folinic acid to be more effective than either alone. This may be suggested by the results shown in Figure 6. However, this patient appeared to be quite sensitive to vitamin B_{12} and the second reticulocyte response may well be due to the vitamin B_{12} alone, unaffected by the folinic acid.

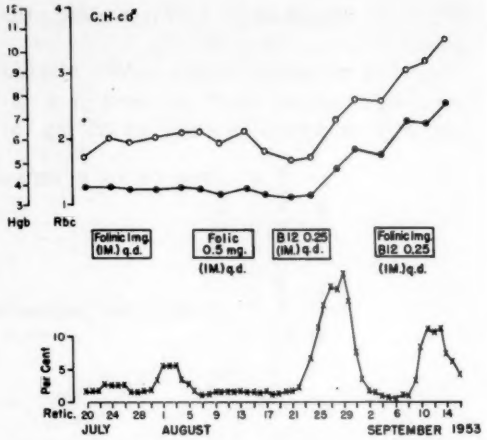

Fig. 6. Comparison of suboptimal doses of vitamin B_{12}, folic acid, and folinic acid in a case of pernicious anemia in relapse.

Therefore, we must admit that the exact significance of the role of vitamin B_{12} in folic acid metabolism is not known. It undoubtedly participates in the breakdown of folic acid conjugate in food to folic acid, as previously mentioned. Further evidence of its importance in this reaction is supplied by experimental data indicating that vitamin B_{12} functions as an activator of enzyme systems containing sulfhydryl groups, among which are the desulfhydrases. One of these is essential for the activation of folic acid conjugase. Vitamin B_{12} may also be important in other steps in folic acid metabolism which we know nothing about at the present time.

ROLE OF ASCORBIC ACID

3. The third proposition is: ascorbic acid is necessary in folic acid metabolism and is in some way related to vitamin B_{12} metabolism.

Figure 7 shows that the plasma level of ascorbic acid is subnormal in patients with macrocytic anemia as compared to a group with similar dietary background but without macrocytic anemia.

Table II reveals that sub-maximal reticulocyte responses and red cell rises occur with ascorbic acid therapy in patient with megaloblastic anemias.

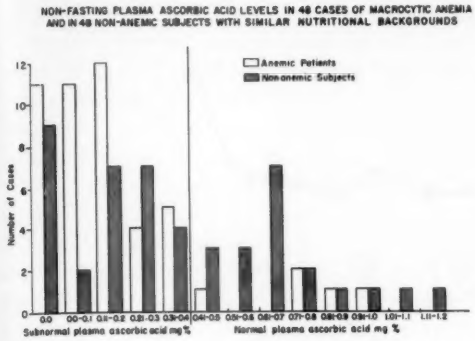

Figure 7

Nichol and Welch[12] drew attention to the possible role of vitamin C when they demonstrated in liver slices the effect of ascorbic acid as a reducing agent in the conversion of folic acid to folinic acid. However, if this reaction were the only one in which ascorbic acid was involved, then one would expect that folinic acid would be more active than folic acid. That this is not true was recorded in Figures 5 and 6.

The importance of ascorbic acid in the folic acid metabolic cycle is evident from the work of May[13] in the megaloblastic anemia induced in the monkey by severe vitamin C deficiency and moderately severe folic acid deficiency. This anemia responds best to folic acid therapy, but also to vitamin C plus vitamin B_{12} treatment. This latter synergism, also observed in the patient with pernicious anemia of pregnancy, suggests that vitamin B_{12} may have something to do with ascorbic these substances effect a common reaction at different levels. An attempt was made to prove this by administering sub-optimal doses of vitamin B_{12} before and after large doses of ascorbic acid and then in conjunction with this vitamin (Figs. 8 and 9).

These results are inconclusive; so we have attempted to approach the problem in another way. At the present time we are in the process of studying the effect of vitamin B_{12} on the oxidation of an intravenously administered dose of reduced vitamin C. Our hypothesis is that in the patient with megaloblastic anemia there may be increased conversion of ascorbic acid to dehydro-ascorbic acid, the oxidation form which can be measured in plasma, and that vitamin B_{12}, by its ability to stabilize SH groups and thus protect other reducing substances such as glutathione or cysteine, might prevent this abnormally rapid

TABLE II

Name	Plasma Ascorbic Acid Mg. %	Ascorbic Acid Therapy(Mg)	Retics (%) Initial	Retics (%) Maximum	Erythrocytes(M) Initial	Erythrocytes(M) Maximum	Hemoglobin(Gms) Initial	Hemoglobin(Gms) Maximum
Alverson(PA)	0.1	500 x 10d	1.8	4.4	1.78	1.75	7.6	7.9
Grammer(PA)	0.1	1000 x 10d	0.8	6.6	2.40	3.18	9.2	12.3
Duke(PA)	0.0	1000 x 12d	1.6	6.4	2.22	2.20	9.2	11.0
Sullivan(PA)	0.1	500 x 10d	1.4	1.2	2.39	1.83	7.2	5.4
Loggins(PA)	0.8	1000 x 10d	1.8	3.0	1.82	2.38	8.6	9.7
Keenam(PA)	0.2	500 x 8d	0.6	3.6	1.50	1.40	4.6	3.6
Goodwin(PA)	0.9	500 x 15d	1.4	3.0	3.16	2.99	10.7	10.0
Gothard(PA)	0.1	500 x 17d	0.4	2.0	1.43	1.16	5.7	5.6
Crump(NMA)	0.2	500 x 42d	1.0	14.8	1.87	4.16	7.7	13.4
Scheinert(NMA)	0.1	500 x 21d	0.2	4.8	2.48	2.03	7.4	4.8
Stearnes(?)	0.0	500 x 16d	-	4.0	1.66	1.70	8.2	9.2
Watkins(?)	0.0	1000 x 16d	0.6	5.5	1.17	1.19	5.2	4.7

12 Persons with Pernicious Anemia or Nutritional Macrocytic Anemia Treated with Ascorbic Acid and a Meat Free B complex poor diet. (Dr. Carl Vilter 1945)

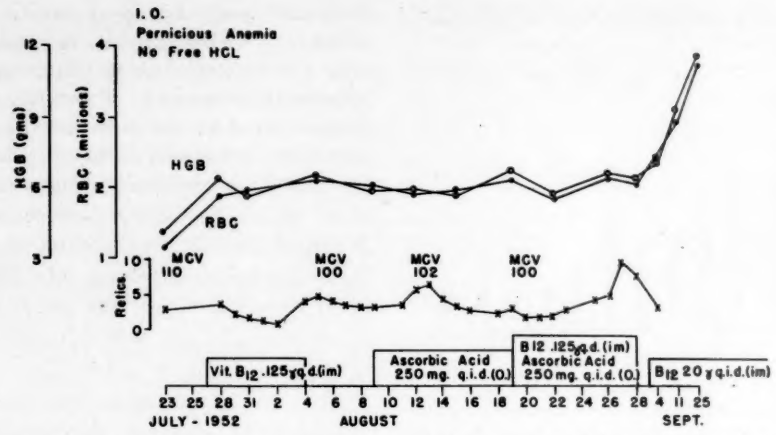

Fig. 8. Ascorbic acid plus suboptimal doses of vitamin B_{12} in a case of pernicious anemia in relapse.

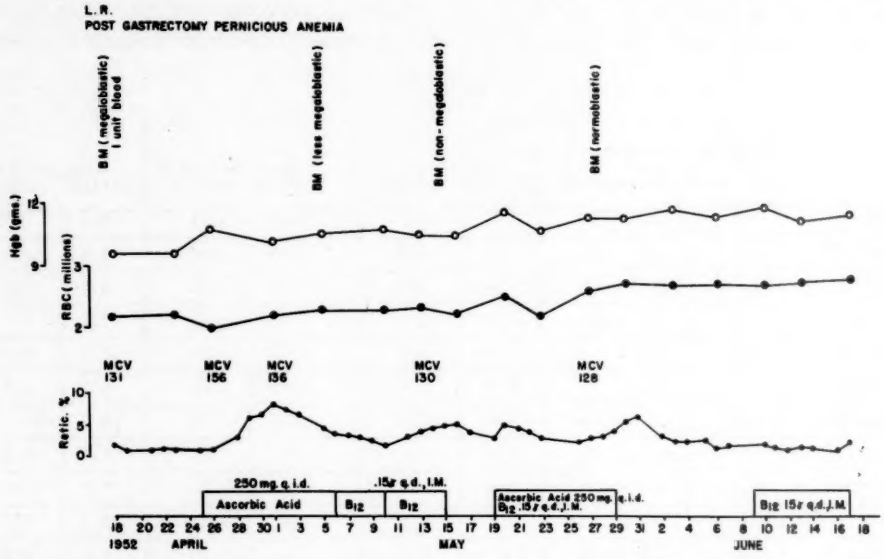

Fig. 9. Ascorbic acid and suboptimal doses of vitamin B_{12} in post-gastrectomy megaloblastic anemia.

oxidation of ascorbic acid. It would appear from our early studies that this is indeed a fact in some patients with pernicious anemia. This rapid oxidation of ascorbic acid also occurs in many other disease states in which a low content of ascorbic acid is found in the body. It does appear, however, that vitamin B_{12} returns the low values to normal before the body stores are replenished from food. Work on this problem is in progress at the present time.

NUCLEOPROTEIN METABOLISM

4. The fourth proposition is: deficiencies of folic acid and vitamin B_{12} in patients with megaloblastic anemias are associated with changes in nucleoprotein metabolism. Vitamin B_{12} and folic acid are necessary in the early steps of nucleoprotein formation, that is, in the formation of open ring purine and pyrimidine precursors from methyl group sources and from nitrogen contained in the body's common nitrogen pool. For this rea-

son, the more complex desoxyribosenucleic acid and ribosenucleic acid components, uracil, thymine, and thymidine, may be active in replacing folic acid and vitamin B_{12} in the treatment of patients with megaloblastic anemias.

Three patients with pernicious anemia in relapse were treated with oral uracil.[14] One case had no hematologic response. One case received 15 grams of uracil daily for ten days. On the ninth day of therapy the patient had a reticulocyte response of 7 per cent. A whole blood transfusion was given inadvertently and no further conclusions could be drawn.

treatment of six cases of pernicious anemia in relapse. The oral administration of approximately ten grams of thymine produced a hematologic response in every way similar to that following the administration of folic acid.

Figure 11 indicates the response to thymine of a case of pernicious anemia in relapse. Doses of 1.0 gram daily were ineffective. When 6 grams a day were given for 12 days a peak reticulocyte response of 14.2 per cent was obtained on the 11th day. The erythrocyte level increased from 2.01 million to 2.88 million cells per cu. mm. and the hemoglobin

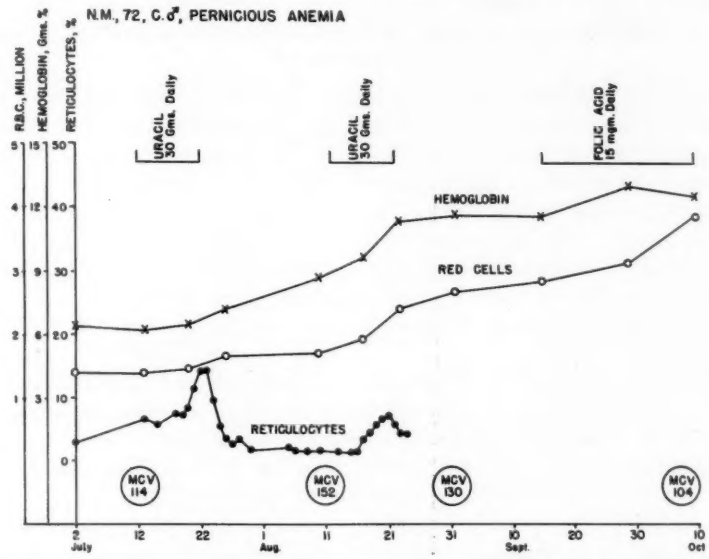

Fig. 10. Effect of uracil in a case of pernicious anemia in relapse (Vilter et al. *Blood* 5: 695. 1950).

The third case (Figure 10) received 30 grams of uracil daily for ten days. On the ninth day of therapy the patient had a reticulocyte response of 15 per cent. A second course of uracil in the same dosage resulted in a second reticulocyte response. The erythrocyte level rose from 1.4 million to 2.8 million cells per cu. mm. and the hemoglobin from 6 grams to 11 grams per 100 ml. During the period of treatment the bone marrow progressively reverted from a megaloblastic to a normoblastic one.

In 1946 Frommeyer, Spies, and Vilter[15] reported the effectiveness of thymine in the from 6.3 grams to 10.4 grams per 100 ml. over a 22-day period. Thymine was then reinstituted and produced a second reticulocyte response, followed by a continued increase in erythrocytes.

Two cases of pernicious anemia in relapse following folic acid therapy were also treated with thymine.[14]

The first case after 13 months of therapy with folic acid developed a hematologic relapse. Twelve grams of thymine daily for 10 days resulted in a reticulocyte peak of 10 per cent on the ninth day of therapy. The erythrocytes rose from 2 million to 3 million

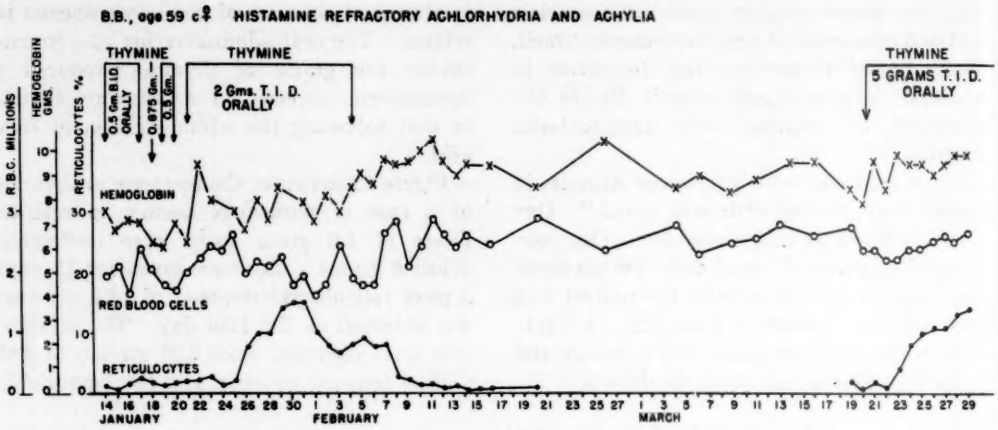

Fig. 11. Effect of thymine in a case of pernicious anemia in relapse (Frommeyer et al. *J. Lab. & Clin. Med.* 31: 643, 1946).

cells per cu. mm. and the hemoglobin from 9 grams per cent to 11 grams per cent. Another case after 32 months of folic acid therapy developed a hematologic relapse. Fifteen grams of thymine daily resulted in a reticulocyte peak of 5 per cent on the eighth day of therapy. No change in erythrocyte or hemoglobin level occurred. The bone marrow was converted from a megaloblastic to a normoblastic type.

Reisner and West[16] reported two cases of pernicious anemia in relapse treated with thymidine in dosage of 5 mg. and 150 mg., respectively, given as a single injection, and one case of pernicious anemia in relapse treated with 5 mg. of thymidine daily for nine days. Reticulocytosis was produced, but no significant increase in erythrocytes occurred. In 1949 Hausmann[17] briefly referred to two cases of pernicious anemia in relapse treated with a total of 2 grams of thymidine given in daily dosage of 100–200 mg. In these cases there was a significant reticulocytosis and increase in red blood cells, as well as a conversion of a megaloblastic marrow to a normoblastic marrow. There has been no further confirmation of these observations.

These results indicate that uracil in dosage of 30 grams, thymine in dosage of 10–15 grams, and thymidine probably in milligram dosage are capable of producing reticulocytosis, some increase in erythrocytes, and conversion of a megaloblastic bone marrow to a normoblastic type in pernicious anemia. These results may be interpreted to indicate that uracil, thymine, and thymidine may be products of reactions catalyzed by folinic acid coenzyme and vitamin B_{12}. Their activity in a patient with pernicious anemia in relapse results either from circumventing the early steps of nucleoprotein metabolism requiring folic acid and vitamin B_{12}, or from a mass action effect of large amounts of these substances in the presence of minimal amounts of folic acid and vitamin B_{12}. The diminished activity of thymine in patients with pernicious anemia relapsing after folic acid therapy, as compared to its activity in spontaneous relapses of pernicious anemia, can be explained by a greater deficiency of vitamin B_{12} induced in these patients by folic acid therapy. This increased vitamin B_{12} deficiency in patients with folic acid relapse is characterized by the heightened neurological manifestations, the hypocellular marrows, and the slow response to vitamin B_{12}.

OTHER ANEMIAS

Similar studies have been carried out in one case of pernicious anemia of pregnancy and in one case of vitamin B_{12}-refractory megaloblastic anemia.[14]

Figure 12 indicates the hematologic response in a case of pernicious anemia of preg-

nancy treated first with uracil, then methionine and choline, and finally thymine. Thirty grams of uracil daily for ten days produced no hematologic response. Methionine and choline in 6- and 3-gram doses daily resulted in a reticulocytosis of 7 per cent on the ninth day of treatment. Little change in erythrocytes occurred. The marrow reverted from somewhat different from that which is responsible for Addisonian pernicious anemia. There is no deficiency of vitamin B_{12}, as is indicated by its therapeutic ineffectiveness. The metabolic defect is probably somewhere in the chain reactions leading to the formation of folinic acid coenzyme. Uracil is inactive in this situation because folinic acid coenzyme

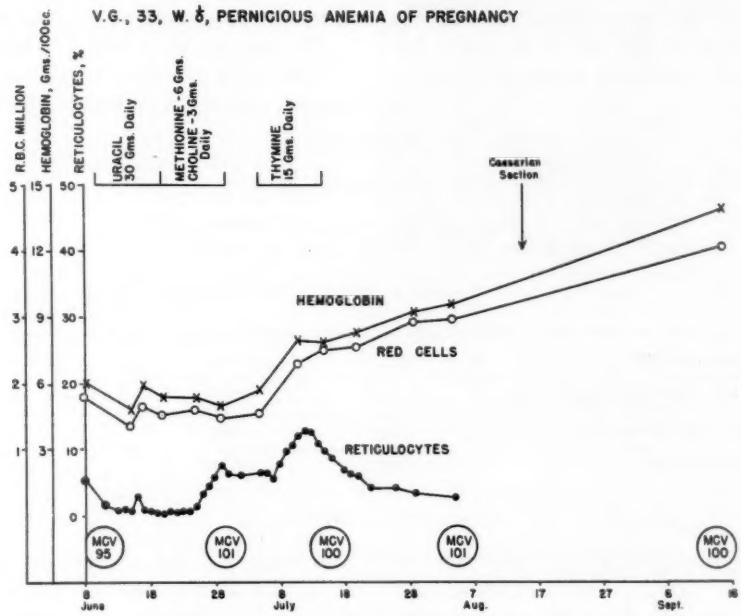

Fig. 12. Effect of uracil, methionine and choline, and thymine in a case of pernicious anemia of pregnancy (Vilter et al. *Blood* 5: 695, 1950).

a megaloblastic to a normoblastic type. Thymine in dosage of 15 grams daily for ten days produced a second reticulocytosis, and the erythrocytes rose to 3 million cells per cu. mm. Similar studies were made on a patient with vitamin B_{12}-refractory megaloblastic anemia. During a relapse, uracil and thymine were administered in dosage of 30 and 15 grams, respectively, for ten days each. Uracil produced no hematologic response. Thymine produced a peak reticulocyte response of 9 per cent, followed by an increase in hemoglobin and erythrocytes.

The observations made on these two patients suggest that the metabolic defect in pernicious anemia of pregnancy and vitamin B_{12}-refractory megaloblastic anemia is is necessary for methylation of uracil-like compounds to form thymine-like compounds. Thymine is capable of producing a hematologic response because it is a product of earlier steps in nucleoprotein metabolism requiring folinic acid coenzyme and vitamin B_{12}, and its further utilization does not require the presence of folinic acid coenzyme. The response to choline and methionine, which contain labile methyl groups, also supports the concept that the defect is in folic acid metabolism, as one of the important functions of folinic acid coenzyme in the early steps of nucleoprotein metabolism is the transfer of single carbon units.

Further information pointing to differences in the activity of folic acid and vitamin B_{12} in

pernicious anemia, and pointing to a relationship of these vitamins to nucleoprotein metabolism was obtained by Horrigan, Jarrold, and Vilter,[18] utilizing the technique of direct instillation of folic acid and vitamin B_{12} into the bone marrow cavity.

The instillation of 1 microgram of vitamin B_{12} into the bone marrow cavity in a case of pernicious anemia in relapse resulted in the maturation of the erythroid cells at the end of 48 hours at the site of injection, but not in the cells aspirated from the opposite hip.

per 100 white blood cells and 100.5 normoblasts per 100 white blood cells. The specimen obtained from the opposite hip showed no significant change from the pretreatment control.

Table IV gives the differential cell count in a typical case of pernicious anemia following the local injection of 1 milligram of folic acid. No maturaiton of the erythroid cells occurred after the local injection of folic acid.

By means of staining techniques with methyl green-pyronin, Horrigan *et al*[18] demonstrated that the cytoplasmic ribosenucleic acid

TABLE III*

STUDIES IN PERNICIOUS ANEMIA

NUCLEATED ERYTHROID CELLS IN BONE MARROW BEFORE AND AFTER LOCAL INSTILLATION OF VITAMIN B_{12}

		Before Vitamin B_{12}	After Vitamin B_{12}	Opposite ilium after Vitamin B_{12}
MEGALOBLAST	per 100 W.B.C.	16.0	3.5	11.0
EARLY ERYTHROBLAST		32.0	15.5	32.5
LATE ERYTHROBLAST		18.0	25.5	14.5
NORMOBLAST		43.0	100.5	43.5

* Horrigan et al. *J. Clin. Investigation* 30: 31, 1951.

Table III gives the differential cell count in a typical case.[16] Before treatment there were 16 megaloblasts and 32 early erythroblasts per 100 white blood cells. Forty-eight hours after instillation of 1 microgram of vitamin B_{12} into the marrow cavity, a sample from the site of injection showed only 3.5 megaloblasts

was condensed and clumped in megaloblastic cells. In similar stained smears, following the local instillation of vitamin B_{12} the

TABLE IV*

STUDIES IN PERNICIOUS ANEMIA

NUCLEATED ERYTHROID CELLS IN BONE MARROW BEFORE AND AFTER LOCAL INSTILLATION OF FOLIC ACID

		Before folic acid	After folic acid	Opposite ilium after folic acid
MEGALOBLAST	per 100 W.B.C.	7.5	8.5	5.5
EARLY ERYTHROBLAST		25.5	23.0	16.5
LATE ERYTHROBLAST		17.5	21.5	16.0
NORMOBLAST		21.5	29.0	31.0

* Horrigan et al. *J. Clin. Investigation* 30: 31, 1951.

stained ribosenucleic acid becomes more diffuse and homogenous, resembling the staining properties of ribosenucleic acid of early erythroblasts from a patient with anemia due

to blood loss. Local instillation of folic acid failed to produce these changes in the staining characteristics of cytoplasmic ribosenucleic acid. Oral and parenteral therapy with folic acid, however, produced the same changes as found with local instillation of vitamin B_{12}.

These observations demonstrate that vitamin B_{12} is capable of being bound and utilized locally by erythropoietic cells and produces maturation of these cells. Vitamin B_{12} therefore does not need to be altered by stomach or liver to produce these changes. Folic acid, on the other hand, is inactive locally. With maturation of the erythroid cells induced by local vitamin B_{12} or parenteral folic acid, a qualitative change in cytoplasmic ribosenucleic acid was demonstrated by the staining technique.

All of these clinical observations, as well as biochemical and microbiological data, implicate folic acid and vitamin B_{12} in the formation of nucleic acids or of their most important constituents, the purine and pyrimidine bases. The following determinations were made in order to follow the changes that took place in the bone marrow nucleic acids of persons with megaloblastic anemia when the hematopoietic vitamins were administered.[19]

1. Cellular ribosenucleic acid (RNA) containing uracil and desoxyribosenucleic acid (DNA) containing thymine were determined by the orcinol and diphenylamine reactions, respectively.

2. Uracil and thymine were determined by a paper chromatographic procedure.

3. Seventeen patients have been studied: ten had pernicious anemia; one had sprue; and six had no hematologic disorder.

Ascending chromatograms were developed in acid butanol for the determination of thymine and uracil (Fig. 13). In this system there is a clear separation of thymine and uracil, while adenine, cystosine, and guanine remain near the base line.

The results reported are expressed as molar ratios of uracil to thymine, thymine to total bases, and RNA to DNA, since this method does not as yet allow us to do more than com-

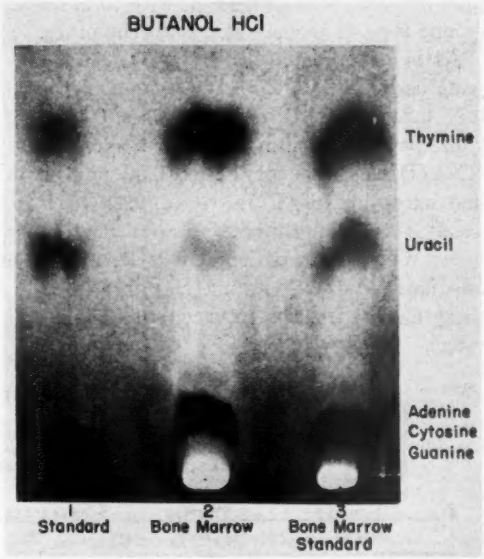

Fig. 13. Ascending chromatogram showing the separation of thymine and uracil from the other purine and pyrimidine bases (Glazer et al. *J. Lab & Clin. Med.* 31: 643, 1946).

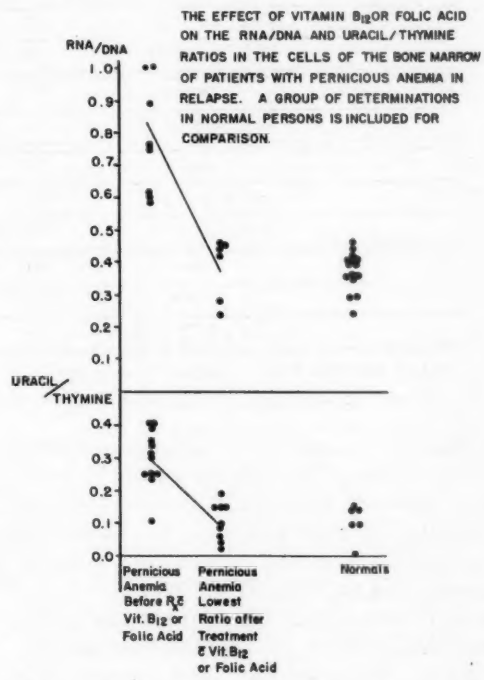

Fig. 14. (Glazer et al. *J. Lab. & Clin. Med.* 31: 643, 1946).

pare one base with another or with total bases, or one type of nucleic acid with the other.

As is indicated in Figure 14, control subjects had RNA/DNA ratios ranging between 0.47–0.23, and U/T ratios between 0.10–0.05, while patients with megaloblastic anemia had RNA/DNA ratios of 1.0 to 0.59 and, with one exception, U/T ratios of 0.41 to 0.26, about three times higher than normal subjects. When these patients were treated with adequate doses of vitamin B_{12} or folic acid, these elevated ratios returned to the normal range.

TABLE V*

Studies of Nucleic Acids in Pernicious Anemia Changes in the Nucleic Acids, Uracil and Thymine of the Bone Marrow and in the Cellular Morphology in Response to Treatment

#	Diagnosis	Therapy	% Megaloblasts	RNA/DNA	U/T	%T
1	Sprue	none	75	1.0	0.27	-
		A.A. 1000 mg x 10	49	0.7	0.16	-
		B_{12} 0.25γ x 10	23	-	0.16	-
		B_{12} 1γ x 10	3	0.33	-	-
2	P.A.	none	74	1.0	0.41	14
		B_{12} 5γ x 3	32	0.65	0.29	20
		B_{12} 5γ x 25	0	-	0.07	26
3	P.A.	none	66	0.76	0.41	-
		B_{12} 15γ x 10	0	0.48	0.16	-
4	P.A.	none	92	0.89	0.37	-
		B_{12} 15γ x 3	54	0.42	0.21	-
		B_{12} 15γ x 10	0	0.43	-	-
5	P.A.	none	80	0.61	0.33	-
		none	88	0.66	0.33	-
		F.A. 1 mg x 10 F.N.A. 1 mg x 10	24	0.48	0.29	-

A.A. = Ascorbic Acid B_{12} = Vitamin B_{12} F.A. = Folic Acid F.N.A.= Folinic Acid

* Glazer et al. *J. Lab. & Clin. Med.* 31: 643, 1946.

Table V demonstrates that these changes in the ratios of RNA/DNA and U/T correlate quite well with disappearance or gradual reduction of megaloblastosis in response to treatment. The first patient with sprue in relapse had an RNA/DNA ratio of 1.0, and a U/T ratio of 0.27 when 75 per cent of the erythroid cells of his marrow were megaloblastic. With ascorbic acid therapy, the RNA/DNA ratio decreased to 0.7 and the U/T ratio decreased to 0.16, while the megaloblasts had decreased to 49 per cent. Subminimal doses of vitamin B_{12} induced further maturation and gradual decrease in these ratios.

The second patient with pernicious anemia was treated with vitamin B_{12} in optimal doses. His initial RNA/DNA ratio was 1.0 and his U/T ratio was 0.41 when 74 per cent of the erythroid cells were megaloblastic. At this time thymine accounted for only 14 per cent of the total purine and pyrimidine bases of the bone marrow. On the third day of vitamin B_{12} therapy, the RNA/DNA ratio had fallen to 0.65 and the U/T ratio to 0.29. Thymine had risen to 20 per cent of the purine and pyrimidine bases, and the bone marrow now contained only 32 per cent megaloblasts. After 25 days on vitamin B_{12}, the bone marrow and the U/T ratio were entirely normal and thymine made up 26 per cent of the total bases. The nine other patients in our series follow a similar pattern. The concentration of thy-

mine rises while that of uracil falls as the bone marrow reverts to normal.

These results provide chemical proof of the importance of vitamin B_{12} and folic acid to nucleic acid metabolism in human beings. They support the biochemical and microbiological data which suggest that vitamin B_{12} and folic acid are involved in the formation of the thymine component of DNA, as well as the cytological observations that these vitamins induce a reduction in the RNA of the pernicious anemia megaloblast.

Summary

Folic acid and vitamin B_{12} deficiency states are associated with defects in nucleoprotein metabolism characterized biochemically by increased ratios of ribosenucleic acid to desoxyribosenucleic acid, and uracil to thymine. These biochemical changes can be correlated with the degree of megaloblastosis. In pernicious anemia, folic acid therapy will temporarily correct the defect in nucleoprotein metabolism by mass action. Eventually, a greater deficiency of vitamin B_{12} occurs, resulting in hematologic relapse and progressive neurologic disease. Folic acid is inactive at the erythroid cellular level, while vitamin B_{12} is active at the erythroid cellular level.

Pernicious anemia of pregnancy and vitamin B_{12}-refractory megaloblastic anemia also occur because of defects in nucleoprotein metabolism. These abnormalities arise because of conditioned deficiencies of folinic acid coenzyme, rather than a deficiency of vitamin B_{12}. Ascorbic acid metabolism may be altered in patients with megaloblastic anemias. The plasma ascorbic acid level is subnormal as compared to a group of patients with similar dietary background but without macrocytic anemia. Vitamin C is capable of producing reticulocytosis and improvement in the anemia of some of these patients. The alteration in ascorbic acid metabolism may reflect changes in redox potentials associated with vitamin B_{12} deficiency.

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The Absorption and Utilization of Vitamin B_{12}

By ROBERT F. SCHILLING, M.D.*

THE HEMOPOIETIC activity of orally administered vitamin B_{12} is enhanced by the simultaneous oral administration of intrinsic factor. The mechanism of this enhanced hemopoietic effect has not been proved, but enhanced absorption of the vitamin has been demonstrated beyond reasonable doubt to occur as one action of intrinsic factor. Another possible explanation of the action of intrinsic factor is the formation with vitamin B_{12} of a complex which is a more potent hemopoietic agent than vitamin B_{12} alone. Intrinsic factor might also retard bacterial utilization of intestinal vitamin B_{12} and thus allow the host to absorb more of the vitamin.

When it was established that vitamin B_{12} was an extrinsic (food) factor as well as an anti-pernicious anemia factor, a logical function to assign to intrinsic factor was the promotion of intestional absorption of the vitamin.[1] Heinle, Welch, Scharf, Meacham and Prusoff[2] demonstrated that intrinsic factor decreased fecal radioactivity in patients with pernicious anemia given radioactive vitamin B_{12} ($B_{12}Co^{60}$) by mouth. A reasonable conclusion was that intrinsic factor enhanced the absorption of the vitamin. Table I is a compilation of data from the literature in which the fecal radioactivity test was used to study pernicious anemia patients for their ability to absorb vitamin B_{12} from the gastrointestinal tract.

The liver has long been known to be a good source of the anti-pernicious anemia factor. The vitamin B_{12} normally in the liver must have been absorbed from the intestine. Glass and co-workers[3] demonstrated that the liver of normal persons who ingest radioactive vitamin B_{12} will contain radioactivity detectable by external scintillation counting, but the patient with pernicious anemia does not demonstrate such radioactivity unless intrinsic factor is given with the ingested radioactive vitamin. These workers have clearly demonstrated an inverse relationship between the oral dose of vitamin B_{12} and the percentage of the dose deposited in the liver, i.e. absorbed.[4] Swenseid and her colleagues[5] have demonstrated a similar limit of absorption of vitamin B_{12} as estimated by the fecal radioactivity technic.

TABLE I

Summary of Fecal Radioactivity Excretion Data in Pernicious Anemia

No. patients	Observations	Intrinsic factor	Per cent radioactivity excreted
34	50	—	72–100
23*	27	added	25–66

* Of the 34 patients above.

Normal subjects who take 1 or 2 μg. of radioactive vitamin B_{12} by mouth will excrete none of the radioactivity in the urine in the following 24 hours unless a large (1000 μg.) "flushing" dose of nonradioactive vitamin B_{12} is injected about the time of taking the oral labeled vitamin.[6] Using this "*in vivo* carrier" technique, 9 normal subjects excreted 7–21 per cent of the orally administered radioactivity in the 24-hour urine, whereas 23 patients with pernicious anemia excreted from 0–2.3 per cent of the radioactivity in the 24-hour urine (Table II).

It is to be noted that 26 subjects with histamine-fast achlorhydria but no other history or signs of pernicious anemia excreted from 3.2–29.6 per cent of the radioactivity in the urine. The radioactivity appearing in the

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TABLE II

Urinary Radioactivity after Oral Vitamin $B_{12}CO^{60}$

No. patients	Status	Gastric acid	Added intrinsic factor	Per cent		
				Minimum	Maximum	Average
9	control	present	—	7.0	21.0	14.7
26	no P.A.*	achlorhydria	—	3.2	29.6	13.0
23	P.A.	—	—	0	2.3	0.6
19	P.A.	—	+	3.4	15.0	9.5
8	Total gastrectomy	—	—	0	1.0	0.2

* P.A. = pernicious anemia.

The figures represent the per cent of orally administered radioactivity which was excreted in the urine collected for 24 hours after ingestion of the radioactive vitamin.

urine in this test has been shown by MacLean and Bloch[7] to have a chromatographic mobility identical with vitamin B_{12}. They also report that 3 normal individuals excreted 20–29 per cent of the orally administered radioactivity in the urine within the first 24 hours, corresponding to 34–39 per cent of the absorbed dose.

Figure 1 is a scattergraph of urine volume versus urine radioactivity, and it is apparent that the two functions are not directly dependent.

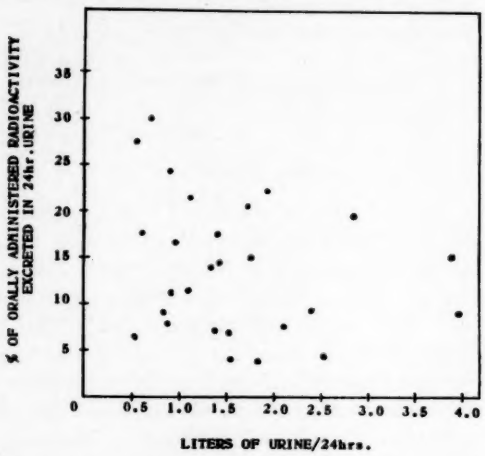

Fig. 1 Scattergraph of urine radioactivity versus urine volume in 27 subjects with histamine-fast achlorhydria but no other evidence of pernicious anemia. Details of technique in text.

Chow[8] has reported data suggesting that elderly subjects might absorb vitamin B_{12} less efficiently than young persons. Figure 2 is a scattergraph plotting age of subject against urine radioactivity after oral radioactive vitamin B_{12}. These data offer no support for the theory that older persons absorb vitamin B_{12} less efficiently than do younger subjects. The data in Figure 2 are evidence that most achlorhydric subjects without other signs of pernicious anemia function as do the control subjects in this test of vitamin B_{12} absorption and excretion.

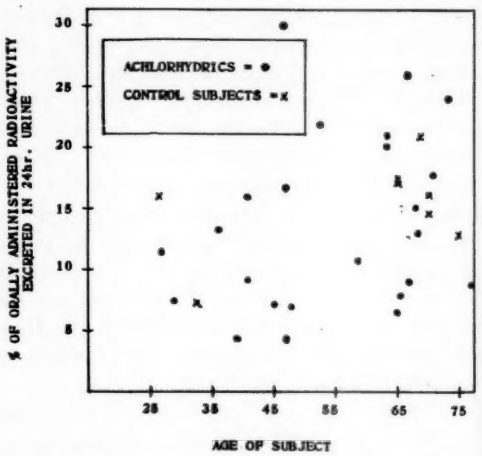

Fig. 2 Scattergraph of urine radioactivity versus age of patient. See text for technique.

As estimated by the urinary excretion of radioactivity, the absorption of vitamin B_{12} by a patient with pernicious anemia was not increased when the intestinal bacterial population was reduced by oxytetracyclene therapy.[6] Thus, as in Ungley's earlier study[9]

using hemopoiesis as an index of vitamin absorption, no evidence was found to suggest that intestinal bacteria were preventing vitamin B_{12} absorption. The observed hemopoietic effects of antibiotics in some megaloblastic anemias[10,11] might be due to increased bacterial production of folic acid or citrovorum factor. As would be anticipated from the earlier work of Castle,[12] Paulson, Conley, and Gladsden,[13] McDonald, Inglefinger, and Belding,[14] studies with radioactive vitamin B_{12} have demonstrated that totally gastrectomized patients are quantitatively similar to patients with pernicious anemia in their inability to absorb vitamin B_{12}[15,16] (Table II). Two of the patients in our series had had end-to-end esophago-duodenostomies, and their vitamin B_{12} absorption was as low as in those patients having an esophago-jejunostomy with a blind loop of duodenum. The data from observations on totally gastrectomized persons also indicate that the stomach is not necessary for vitamin B_{12} absorption if intrinsic factor is supplied. The small intestine may be considered the site of absorption of vitamin B_{12}.

The vitamin B_{12} content of the feces from a patient with pernicious anemia in relapse is more than adequate to treat him by injection.[17] Patients with pernicious anemia in relapse have been treated by intramuscular injections of vitamin B_{12} extracted from their own feces.[18] If intrinsic factor is given 12 hours before the oral vitamin B_{12}, the hemopoietic activity of the vitamin will not be increased.[1] From this one might conclude that the intrinsic factor is inactivated during this period, or, possibly, when the intrinsic factor has reached the colon, it can no longer effect the absorption of vitamin B_{12}. Evidence for the latter postulate is the observation by Best and colleagues,[19] who found that an enema of radioactive vitamin B_{12} and intrinsic factor did not lead to urinary excretion of radioactivity after a "flushing" injection of nonradioactive vitamin B_{12}.

The poor absorption of vitamin B_{12} observed frequently in sprue is not corrected by adding intrinsic factor.[3]

If massive oral doses of vitamin B_{12} (1000–5000 μg./day) are given to patients with pernicious anemia in relapse, excellent hemopoietic responses will be observed in the absence of added intrinsic factor.[9] Nasal instillation and aerosol inhalation of vitamin B_{12} have been reported as effective modes of therapy for pernicious anemia.[20,21] Intramuscular injections of vitamin B_{12} at three- to four-week intervals in amounts equal to 1 μg./day have been found to be satisfactory for maintenance therapy.[22]

Latner and his colleagues[23] have reported the "isolation of the intrinsic factor." At the oral dose level of 1 mg., their material decreased the fecal radioactivity in patients with pernicious anemia given radioactive vitamin B_{12}. By electrophoretic and ultracentrifugal study this preparation was considered to be pure. In unpublished observations by the author a hog mucosal concentrate* was found to be active at 1 and 2 mg. levels in the urinary radioactivity assay for intrinsic factor.

Several chemical forms of vitamin B_{12} in addition to cyano-cobalamin are known.[24,25] Hydroxo-cobalamin[25,26] and nitro-cobalamin[25] (vitamin B_{12c}) possess anti-pernicious anemia activity when injected. Hydroxocobalamin will also serve as an extrinsic factor;[26] hence any postulated chemical bond between extrinsic and intrinsic factors cannot be dependent upon the specific cyanocobalamin configuration. Pseudo-vitamin B_{12} contains an adenine moiety instead of the 5,6-dimethyl benzimidazole of the usual cobalamins, and it is reported to lack anti-pernicious anemia activity.[27]

Vitamin B_{12} which is absorbed has a slow turnover rate. The prolonged remissions[28] which sometimes persist in patients with pernicious anemia after therapy with B_{12} is discontinued suggest this. Direct observations of hepatic radioactivity after therapy with radioactive vitamin B_{12} show that much of the activity remains in the liver for more than one year. That excessive metabolic activity may increase the need for vitamin B_{12} is suggested by the fact that there are in the literature at least 75 instances of the co-

* Supplied to the author by W. F. White of Armour Laboratories.

existence of hyperthyroidism and pernicious anemia.[29] The author feels that this figure is higher than would be anticipated from chance alone: statistical proof, however is not available. The hypermetabolic rat has an increased vitamin B_{12} requirement.[30]

The classical human example of vitamin B_{12} deficiency is that caused by an unexplained lack of intrinsic factor activity, pernicious anemia. The parenteral injection of vitamin B_{12} will completely correct the hematologic defects of this disease, and the neurologic defects will be halted or reversed. The sore tongue will disappear. Lajtha[31] and Thompson[32] have reported independently that vitamin B_{12} added *in vitro* to cultures of marrows from pernicious anemia patients did not cause a maturation of the megaloblasts. Folic acid, however, did cause such a maturation *in vitro*. When vitamin B_{12} was added with intrinsic factor, maturation occurred. Horrigan, Jarrold, and Vilter[33] demonstrated that local *in vivo* instillation of vitamin B_{12} into one iliac marrow caused local maturation of that marrow in 24 hours, but not of the marrow in the opposite ilium of the patient with pernicious anemia. Wallerstein and colleagues[34] found no consistent evidence that intravenous B_{12} and gastric juice was a more effective hemopoietic combination than intravenous vitamin B_{12} alone.

The fact that the cells from the vaginal and gastric mucosa in pernicious anemia in relapse are macrocytic and often multinucleated[35] is evidence that vitamin B_{12} is required for normal development of cells from several systems in addition to the hemopoietic and neurologic.

Summary

The absorption of vitamin B_{12} in the small intestine from the dietary intake requires an adequate supply of intrinsic factor. In man, the only apparent gastrointestinal source of intrinsic factor is the gastric mucosa. Less than 1 μg./day is the normal requirement of absorbed vitamin B_{12}. The total daily dietary requirement is not known. There is evidence that the biologic rate of decay of this vitamin is relatively slow.

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DISCUSSION

DR. J. R. KREVANS (Baltimore, Md.): I'd like to make just a few very brief comments about Dr. Schilling's excellent paper, and ask some questions.

We got very much the same results that Dr. Schilling did with respect to age, using the technique of feeding a patient a certain amount of labeled vitamin B_{12} and recovering radioactivity in the feces. We feel that this adds strength to the argument. At least, by these two techniques, age does not seem to be an important factor in the percentage of B_{12} absorbed, and I think our method of study helps to strengthen that argument because it bypasses the question of whether or not the elderly individual's kidneys are working as well as the young adult's.

Second, we were able to confirm the observations about the inability of terramycin to improve the absorption of vitamin B_{12} in patients with pernicious anemia.

We had an opportunity to study an indivi-

dual who had a vitamin B_{12} deficiency on another basis, multiple small intestinal diverticulae, and who had neurologic changes. Now, this individual's B_{12} absorption was very definitely improved by the prior administration of terramycin, suggesting that at least in this situation bacterial competition played an important role.

This observation is confirmed by a very interesting case report by Scandinavian workers, who reported a patient with multiple intestinal strictures and fistula who had pernicious anemia, and who had a clinical and hematological response to the administration of terramycin rather than vitamin B_{12}. I think it is worth pointing out that not all patients with sprue have an absorption defect for vitamin B_{12}, and I think both Dr. Glass and our group have found some individuals with a full-blown picture of sprue who are able to absorb vitamin B_{12} perfectly normally.

Finally, we have been able to follow some 50 patients now, with pernicious anemia, who have been treated with orally administered vitamin B_{12}. Ten of these patients have been started and maintained on orally administered B_{12}, and some 20 patients have been started on oral B_{12} but not maintained on this therapy. Others have been maintained on oral B_{12}, although they had been started on liver extract or parenteral vitamin B_{12}. The longest any of these patients has been maintained on oral B_{12} has been since the winter of 1950. The patients received initially five milligrams of vitamin B_{12} orally, without any gastric juice or intrinsic factor, and are maintained on one milligram per week. To date, we have had no clinical or hematological relapses on this schedule.

I would like to ask Dr. Mueller this question: Do those patients with pernicious anemia who were maintained on folic acid for long periods of time, and who eventually relapsed, show a picture of hypoplasia in the bone marrow, and no megaloblastic changes? I'd like to hear this discussed further. It is so convenient to think of the megaloblastic picture as part of the same general B_{12} deficiency state that causes immature cells to be found in the gastric mucosa, and I wonder why these individuals do not have megaloblastic marrows when all their B_{12} has been consumed?

DR. J. F. MUELLER (Cincinnati, Ohio): The observation has been repeatedly noted that a hypocellular nonmegaloblastic bone marrow is present in patients who are treated for months or years with folic acid. We interpret this finding as showing a severe B_{12} deficiency, certainly with no folic acid deficiency. It fits in well with the published reports of experiments with swine. In these animals pure dietary B_{12} deficiency did not produce megaloblastic changes in the bone marrow, whereas folic acid deficiency did.

DR. D. L. HORRIGAN (Cincinnati, Ohio): Certainly Dr. Will's data on the different ratios in pernicious anemia marrows are very interesting. I wonder how much of that might be due to simple immaturity of the marrow. And I wonder if he has any data on an iron deficiency marrow or one that is hyperplastic due to acute hemolytic anemia, to show that the immaturity itself is not responsible for these changes.

DR. J. J. WILL (Cincinnati, Ohio): Yes, we feel this is not evidence of mere immaturity, but is a specific change that took place in the megaloblastic type cells.

DR. B. CONNOR JOHNSON (Urbana, Ill.): As many of you know, we have been working with vitamin B_{12} deficiency, using the baby pig as experimental animal, for the past five years. B_{12}-deficient baby pigs do not show a megaloblastic anemia, even though they do die of the deficiency, usually within two to four weeks.

A severe choline deficiency can also readily be produced in the baby pig. As in the rat, this choline requirement can be replaced by dietary methionine. In order to study the interrelationship between this choline requirement and vitamin B_{12}, a series of *in vivo* and *in vitro* experiments have been carried out. The first group of experiments were designed to find out whether vitamin B_{12} is involved in direct transmethylation reactions. *In vivo*

it was found that baby pigs on a B_{12}-deficient, choline-free diet, containing sufficient methionine to provide both sulfur amino acid and methyl requirements of the animal, were able to transmethylate from this methionine to make choline for prevention of fatty livers just as well as were B_{12}-adequate animals. There was no evidence of choline deficiency even in animals dying of B_{12} deficiency in this experiment. This fact, that B_{12} is not involved in direct transmethylation from methionine to form choline, was confirmed in *in vitro* studies with liver homogenates of B_{12}-deficient as compared to normal pigs and chickens. Differences in direct transmethylating ability were found in rats, on the other hand, and may be related to apoenzyme formation, or to some other effect, I am not sure. I would be interested in hearing Dr. Williams' comments on this.

Since we didn't find vitamin B_{12} involved in transmethylation, we of course examined methyl synthesis, following the line of approach used by Dr. Stekol and Dr. Arnstein. In *in vivo* experiments we fed baby pigs glycine as a possible methyl precursor. On choline-free diets containing only enough methionine to satisfy the animals' needs for sulfur amino acids, the B_{12}-deficient pigs on these diets all showed a severe choline deficiency; however, with vitamin B_{12} added, there were no fatty livers and no evidence of choline deficiency, indicating, therefore, by a straight nutrition experiment, that B_{12} is required for methyl synthesis from, in this case, glycine; that is, in the presence of vitamin B_{12} glycine completely replaced choline in the diet. This was substantiated by tracer experiments in these pigs in which the C^{14} of α-labeled glycine was found to be incorporated into the methyl groups of choline in the presence of B_{12}, while only to about a tenth as much in the absence of vitamin B_{12}.

DR. J. N. WILLIAMS (Madison, Wis.): These experiments actually seem to point up exactly what I was trying to bring out this morning: that there are many reactions in some way associated with folic acid and vitamin B_{12}, but the *exact* steps at which the vitamins are involved cannot be stated with any certainty. An easy explanation is that in the case of the pig one is dealing with a different type of enzyme than in the rat. However, I wouldn't like to make that statement without equivocation. I am able to think of an example of an enzyme that is different in its mechanics of action in one animal as compared to another. That is xanthine oxidase in the rat as compared to xanthine dehydrogenase of the chick. That is one possibility, but beyond that I have no other explanation.

Relationships of Hormones *to the Utilization of* Essential Nutrients *in* Erythropoiesis

By ROGER C. CRAFTS, PH.D.*

CARTWRIGHT[1] divided the known factors concerned in erythropoiesis into vitamins, amino acids, and minerals. In his survey he reported that the following vitamins played a role in this process in at least one animal species, although not necessarily in the human being: riboflavin, nicotinic acid, pyridoxine, *Lactobacillus casei* group, extrinsic factor (vitamin B_{12}), ascorbic acid, pantothenic acid, and biotin. The globin fraction of the hemoglobin molecule is known to contain many of the so-called essential amino acids and many of those called nonessential. Of the amino acids, tryptophane, lysine, phenylalanine, isoleucine, and glycine have received greatest attention and anemias have been described as resulting from deficiencies in these substances. Three minerals —iron, copper, and cobalt—have been shown to be essential for normal erythropoiesis.

Except for the well-known role the endocrines play in general metabolism, a discussion of which is not the purpose of this paper, there is very little known about the specific role of the hormones in the metabolism of these essential nutrients in erythropoiesis. On the other hand, many of these substances have been used in a therapeutic manner in an attempt to prevent or alleviate anemias which have been induced by the removal of the endocrine glands. This paper, therefore, will discuss the problem from this point of view.

The anemia induced by the removal of the hypophysis has received greatest attention. This anemia, in rats, is characterized by a hypoplasia of the bone marrow with a decrease in erythroid elements, a 30 per cent decrease in erythrocyte count, a 33 per cent decrease in hematocrit, a 31 per cent decrease in hemoglobin, a slight decrease in mean corpuscular volume, a slight decrease in mean corpuscular hemoglobin, and a normal mean corpuscular hemoglobin concentration.[2] If the hemoconcentration which occurs after hypophysectomy is taken into account, the anemia is actually more severe.[3] This anemia has been found in every experimental animal studied, and human beings with panhypopituitarism exhibit a similar blood picture.[4]

Of the known causes for such an anemia, inadequate iron intake, faulty iron metabolism, and hypothyroidism seemed the only logical ones to investigate.

Daily injections of 0.5 mg. of ferrous sulfate into hypophysectomized rats had beneficial effects, but did not prevent the anemia.[5] Daily injections of 0.025 mg. of cupric sulfate in addition to the iron induced results similar to those obtained with iron alone.[5] Further investigations showed that in spite of the fact that there was a decrease in gastric acidity[6] and a decrease in serum iron[7] after hypophysectomy, there was a normal or elevated amount of iron in storage.[7] Therefore, iron is available in the hypophysectomized animal for hemoglobin formation.

Daily injections of 0.01 mg. of thyroxine into hypophysectomized rats prevented the decrease in erythrocyte number but did not prevent the decrease in hemoglobin.[5] A combination of thyroxine, iron, and copper induced slightly better but similar results.[5]

Androgen injections have been found to induce an increased erythropoiesis in rats,[8–11] in fowl,[12–15] in hamsters,[16] and in the human.[17,18] Daily injections of 2.0 mg. of testosterone propionate into hypophysectomized rats prevented the decrease in erythrocytes

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but did not prevent the decrease in hemoglobin,[19] thus producing results simliar to those after thyroxine therapy.

Because of the difficulty in maintaining a normal hemoglobin level in the hypophysectomized rats, and because protein metabolism is not normal in such animals, it was thought that the globin factor of hemoglobin might be involved. Accordingly, hypophysectomized rats were fed a high protein diet and given 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate daily to aid in the utilization of that diet.[20] With this combination a normal hemoglobin level as well as a normal erythrocyte count was maintained.

Growth hormone, on the other hand, will not prevent posthypophysectomy anemia even in doses which are adequate to maintain growth in hypophysectomized rats.[21] Similar negative results have been reported by others.[22,23] Because of these negative results, it was thought that the good results obtained with the combined thyroxine, androgen, high protein diet therapy might have been due to the thyroxine and androgen only. Further experimentation, however, showed that although thyroxine and androgen would prevent posthypophysectomy anemia, better results were obtained if a high protein diet was added.[24] These results have led some to conclude that faulty protein metabolism is the cause for posthypophysectomy anemia.

Previous work[25] had shown that thyroidectomy induced an anemia which was not as severe as that after hypophysectomy, and that adrenalectomy induced a temporary anemia. This has been confirmed by others.[26,27] Combined thyroidectomy and adrenalectomy, however, induced an anemia which was similar to that found after hypophysectomy in Wistar rats.[28] In addition, it was found that thyroxine and cortisone therapy would prevent posthypophysectomy anemia.[29] Thus, it was concluded that posthypophysectomy anemia might be due to a combined hypothyroidism and hypoadrenocorticalism. This work has been challenged by Van Dyke *et al.*[30] because they were able to repeat the results obtained by a combined thyroidectomy and adrenalectomy, but found that the anemia after hypophysectomy was more severe. This work was performed on younger rats and of the Long-Evans strain. Work is now in progress on a comparison of the effects of hypophysectomy and of combined thyroidectomy and adrenalectomy on erythropoiesis in Wistar, Long-Evans, and Sprague Dawley rats.[31]

Before leaving this aspect of the problem, it should be mentioned that the idea of an erythropoietic hormone being released by the pituitary has been introduced. This idea was first published in 1938 by Flaks, Himmel and Zlotnik.[32] They made extracts of sheep pituitaries which were free of other pituitary factors, and were able to prevent posthypophysectomy anemia by oral administration of this extract. This work was repeated by Contopoulos *et al.*,[33] and although they could not confirm these observations with sheep extracts, they did obtain similar results with extracts of cattle pituitaries. Because of the many objections raised as to the effectiveness of orally administered pituitary hormones, the latter workers have prevented posthypophysectomy anemia with this same extract given intramuscularly.[34] Thus, we have the very interesting contradiction in the literature of data which indicate that the pituitary is responsible for posthypophysectomy anemia in a direct manner (erythropoietic hormone) and other data which implicate the thyroid and adrenal glands.

An increased erythrocyte destruction would not seem to be the cause for posthypophysectomy anemia, in spite of the fact that there is a great increase in the concentration of splenic iron after hypophysectomy. This increase seems to be due to the marked decrease in size of the spleen in these animals. The total amount of iron is normal. In addition, splenectomized-hypophysectomized rats exhibit the same anemia as rats hypophysectomized only, and there is no increase in serum bilirubin after hypophysectomy.[35]

Amazing results have been obtained with cobalt in hypophysectomized rats.[36,37] This mineral not only prevented posthypophysectomy anemia but actually increased the erythrocyte count from a normal of 8.00 mil-

lion cells per cu. mm. to almost 12 million cells; the hemoglobin from 15.8 Gm. per 100 cc. to 21.0 Gm. Because of the finding that vitamin B_{12} contained cobalt,[38-40] hypophysectomized rats were injected with this vitamin and others with liver extract.[36] Neither of these substances had any effect on posthypophysectomy anemia.

In conclusion, there is no doubt that the endocrines have a secondary influence on the essential nutrients for erythropoiesis through their influence on general metabolism. Whether they have a direct influence on these nutrients is still problematical.

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Antimetabolites and Antibiotics as Tools for Research on Blood Formation

By THOMAS H. JUKES, PH.D.

I. ANTIMETABOLITES

STUDIES OF the effect of antimetabolites on blood formation have been pursued by a number of investigators in recent years. Important contributions to this field were made in the late 1940's, and active investigations are still in progress. The nature of the topic makes it necessary to devote some time to a review of the earlier work, in which many of the participants in this symposium played an active and prominent role .

The effects of folic acid deficiency in experimental animals were extensively investigated during the years before this vitamin was known in the pure state, and it was well established that a deficiency led to the production of anemia in various experimental animals and human beings. Accordingly, when the synthesis was achieved,[1] we promptly embarked on a program of making chemical analogues of folic acid with the hope of producing antagonists. It was expected that such antagonists would provide new experimental approaches to the study of blood formation.

In 1946 Hultquist and Smith carried out a folic acid synthesis reaction in which one of the intermediates had an extra methyl group. This intermediate was 2,3-dibromobutyraldehyde, as compared with 2,3-dibromopropionaldehyde which is used in the synthesis of folic acid. A crude reaction mixture was obtained which had properties antagonistic to those of folic acid. Its effects on rats were examined and, in order to forestall its possible reversal by dietary folic acid, the animals were maintained on a purified diet without folic acid and containing one per cent sulfasuxidine. Such diets are known to produce a mild deficiency of folic acid in rats. The precaution of using a restricted diet was later shown to be unnecessary, as the antagonist, x-methyl folic acid, produced an acute deficiency of folic acid in rats even when the diet contained the vitamin, and the deficiency could be reversed by adding folic acid to the diet. The findings were described by Franklin and co-workers in 1947.[2] The deficiency was marked by a slowing of growth, a reduction of the per cent of hemoglobin, a similar but more marked reduction of the white cell count, and, in the differential white count, a greater reduction in the granulocytes than in the lymphocytes. Parallel changes were found in the bone marrow; the maturation of cells of the erythroid series and the production of mature granulocytes were seriously impaired. The findings are illustrated in Table I.

These experiments with rats led us to express the hope that in the crude antagonist we had a substance which could be used in the management of blood dyscrasias such as leukemia and polycythemia. These hopes were not realized, for Welch and Heinle showed that the crude antagonist had little or no effect on human patients even when administered in large doses, several grams daily. Evidently there are marked differences between species in their tolerance of folic acid antagonists, and we have some clues to the possible biochemical mechanisms which may underlie such differences. The crude antagonist was found to produce a well-marked deficiency of folic acid in chicks (Table II).

This deficiency was reversed by folic acid. It was also found that dogs developed a

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TABLE I

Effect of Pteroylglutamic Acid (PGA) and Antagonist on Blood Count of Rats Fed Purified Diet plus Sulfasuxidine

Supplement per Kg. diet		PGA excess or deficit over basal†	Hemoglobin		White count		Granulocytes	
PGA	Antagonist		3 wks.	5 wks.	3 wks.	5 wks.	3 wks.	5 wks.
mg.	*Gm.*							
0	10	−3.3	10.5	*	0.8	*	8	*
1.0	10	−2.3	12.2	*	2.5	*	100	*
0	1.0	−0.3	13.6	10.5	2.8	0.8	84	16
0.3	1.0	−0.03	13.1	18.1	7.8	13.3	470	540
0	0	0	15.1	18.3	8.2	12.6	1500	1500
1.0	0	1.0	16.6	19.3	10.5	15.2	2400	2600
3.0	1.0	2.7	15.1	20.9	10.9	13.7	1500	1100
10	10	6.7	15.1	20.2	9.5	14.2	1200	1100
30	1.0	29.3	15.6	21.3	11.3	14.9	1800	1500
100	10	96.7	15.7	20.6	15.5	18.2	2900	1600

* All dead. † Assuming 3 Gm. antagonist "reverses" 1 mg. PGA.
Hemoglobin: Gm. per 100 ml. White Count: × 1000/cu. mm. Granulocytes: per cu. mm.

TABLE II

Effect of "Crude Methyl Pteroylglutamic Acid" on Growth and Hemoglobin Formation in Chicks

Diet used: Purified basal deficient in pteroylglutamic acid (PGA)

Exper. No.	Supplement per Kg. of diet		Number of chicks per group	Weight and (survivors) at 28 days	Hemoglobin at 28 days	White cell count at 28 days
	PGA	Antagonist				
	mg.	*mg.*		*Gm.*	*Gm. per 100 ml.*	*Cells/cu. mm.*
1	0	0	11	116 (11)	6.3	—
1	0.1	0	10	177 (10)	8.2	—
1	1.0	0	10	310 (10)	10.0	—
1	0.1	1,000	6	95 (2)	4.7	—
1	10.1	1,000	6	353 (6)	9.6	—
2	0	0	10	166 (7)	7.7	12,600
2	1.0	0	10	321 (9)	9.1	19,900
2	1.0	10,000	10	106 (3)	8.4	6,000

marked deficiency of folic acid when the crude antagonist was added to their diet. The deficiency was characterized by anemia and loss in weight, but the effect on the white count was less definite. The deficiency was abolished by adding folic acid to the diet, and the hemoglobin and red cell values returned to normal with a reticulocyte peak on the seventh day as shown in Tables III and IV. The findings in these studies with dogs are summarized in Table V.

In the meantime, the search for more potent antagonists of folic acid had continued, and in 1947 the synthesis of aminopterin was described by Seeger, Smith, and Hultquist.[3] This compound produces markedly toxic effects on most species and its action on animals is not

TABLE III

Production of Pteroylglutamic Acid Deficiency in the Dog by "X-Methyl Pteroylglutamic Acid"

Depletion diet: Purified diet plus 10 Gm. antagonist per Kg. of diet

Recovery diet: Purified diet plus 10 Gm. antagonist plus 100 mg. pteroylglutamic acid per Kg. diet

tion of treatment Nature and dura-	Body wt.	globin Hemo-	RBC	White cells	Retic- ulocytes
	Kg.	*Gm. per 100 ml.*	*Cells/ cu. mm. × 10^6*	*Cells/ cu. mm. × 10^3*	*%*
Depletion diet					
Initial	9.4	16.7	6.7	9.3	
After 14 weeks	6.4	13.5	3.7	11.7	0
Recovery diet					
1 day	6.4	12.5	3.4	8.1	0
7 days	7.3	12.7	4.2	17.8	3.9
21 days	8.6	13.0	4.5	9.1	0.3

TABLE IV

Reversal of Deficiency Syndrome in Dog with PGA

	Original values	Days								
		0	2	4	5	6	7	14	21	28
Weight (Kg.)	9.4	6.4	6.6	6.6	7.0	7.2	7.3	7.9	8.6	8.7
Hemoglobin (Gm./100 ml.)	16.7	13.5	10.6	11.2	12.8	13.8	12.7	14.0	13.0	16.0
Red cell count (cells/cu. mm. $\times 10^6$)	6.7	3.7	3.3	3.8	4.3	4.4	4.2	4.2	4.5	6.2
White cell count (cells/cu. mm. $\times 10^3$)	9.3	11.7	8.4	11.5	18.0	22.0	17.8	14.7	9.1	12.2
Reticulocytes (%)		0	0.8	0.9	3.5	3.7	3.9	1.6	0.3	0.4

Dog 4 was used for this experiment. After a severe deficiency had been established the diet was modified by adding 100 mg. PGA/Kg. diet. The level of antagonist (10 Gm./Kg.) was not changed.

TABLE V

Characteristics of an Antagonist-Induced Pteroylglutamic Acid Deficiency in Dogs

Antagonist used: "X-Methyl Pteroylglutamic Acid"

1. Pteroylglutamic acid-deficient diet *per se* does not produce deficiency symptoms.
2. Deficiency symptoms with antagonist develop more slowly in dogs than in rats or chicks.
3. Loss in appetite and weight with severe dehydration.
4. Skin changes: alopecia, urticarious dermatitis, ulceration of bony prominences.
5. Blood dyscrasia: mild macrocytic anemia, slight leukopenia.
6. No marked diarrhea or gingivitis.
7. Deficiency does not respond to refined liver extracts in a consistent manner.
8. Reversal of deficiency produced by pteroylglutamic acid in the presence of an antagonist with (a) recovery of weight and appetite; (b) disappearance of dermal lesions; (c) restoration of normal blood morphology.

reversed by folic acid. These findings were typified by our report which described results with mice.[4]

The effects of aminopterin on hemopoiesis in experimental animals were studied in detail by Thiersch and Philips.[5] They described a syndrome in mice which included loss of weight; hypoplasia and liquefaction of the bone marrow; and intestinal lesions with diarrhea. The syndrome was rapid in onset and led quickly to death. In studies with mice, we noted that the toxicity was reduced only to a slight extent by folic acid,[6] but that leucovorin or tetrahydro PGA would protect mice against the syndrome.

Thiersch has described the profound effects of aminopterin on the bone marrow of human patients; these included a general depletion of the marrow affecting both the myeloid and red cell series. Recovery was spontaneous after the withdrawal of the drug and was accelerated when folic acid acid was given.[7] Aminopterin was evidently a substance which produced effects on the hemopoietic system beyond those which could be brought about by x-methyl folic acid.

One of the first biochemical clues as to the mechanism of action of folic acid came from Shive and his group,[8] who agreed that folic acid acted as a carrier of a single-carbon fragment in adding the final carbon atom to the purine ring. It is now known that folic acid before participating in certain biochemical reactions must be reduced and formylated to acquire an extra carbon atom. This transformation is blocked by the folic acid antagonists. In addition, aminopterin and its derivatives can block the action of citrovorum factor, which certain other folic acid antagonists typified by x-methyl folic acid seem unable to do. These biochemical considerations are reflected in studies with mice which were carried out by Burchenal and coworkers[9] (Fig. 1). Mice implanted with leukemia AK 4 were found to succumb in about two weeks. Their survival time was prolonged to about four weeks by administering an aminopterin derivative. The protective effect of the aminopterin derivative was abolished by citrovorum factor, but folic acid was ineffective. This indicates that the aminopterin derivative reversibly an-

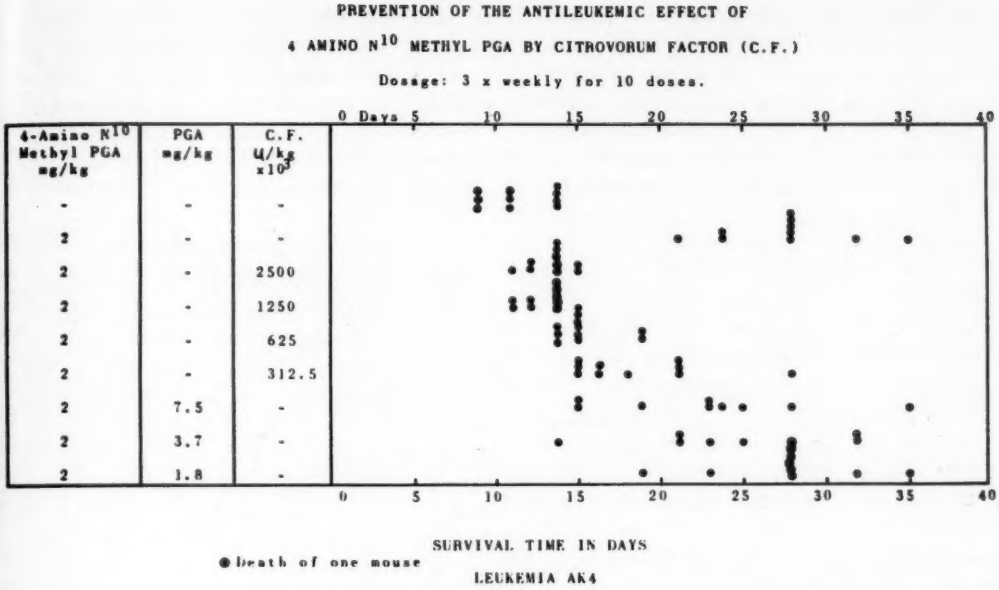

Figure 1.

tagonized citrovorum factor, and that citrovorum factor was concerned in the acceleration of the leukemic process. Folic acid was ineffective because its conversion to citrovorum factor was blocked by aminopterin.

Unfortunately, leukemia in children soon becomes resistant to treatment with aminopterin and its derivatives when these drugs are used for prolonged periods. The mechanism of this acquired resistance has been studied by Burchenal and co-workers, by Welch and Nichol, and by our group using *Streptococcus faecalis* as a model system. This organism can transform folic acid into citrovorum factor in small yield, provided that a source of the formyl group, such as serine or formate, is added to the medium and anaerobic conditions are maintained by some suitable means such as ascorbate. If, however, *S. faecalis* is first grown for several transfers on a medium containing aminopterin or one of its derivatives, a resistant strain is produced. This strain is found to possess a greatly increased ability to form citrovorum factor from folic acid, as illustrated in Table VI. It is therefore possible that the resistant leukemic cell has undergone a similar change in the presence of aminopterin, so that the cell is now able to produce large quantities of citrovorum factor and thus combat the inhibitory action of aminopterin.

TABLE VI

Enzymatic Formation of Citrovorum Factor (CF) from Pteroylglutamic Acid (PGA) by Resting Cells of an Aminopterin-Resistant and Parent Strain of *S. faecalis*

Flask No.	Additions per flask			Total CF formed by	
	5 mg. cells	1 μg. PGA	5 mg. ascorbate 10 mg. formate	Parent strain	Resistant strain
				μμg.	μμg.
1	+	−	−	0.6	0.8
2	+	−	+	1.2	0.6
3	+	+	−	0.7	24
4	+	+	+	6.8	540
5	Boiled	+	+	0.6	0.6

The biochemical and hemopoietic findings with the folic acid antagonists have far-reaching implications in the biochemistry of hemopoiesis because there is excellent evidence that folic acid is concerned with the formation of nucleic acids and hence with cellular proliferation. This is illustrated in Figure 2

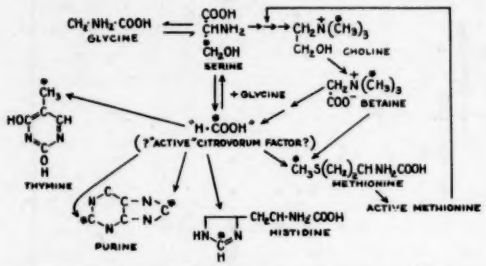

Fig. 2. Biochemical changes involving the "single-carbon unit."

which indicates a catalytic role for folic acid ("active citrovorum factor") in the formation of purines and thymine and in other biochemical reactions. These biochemical relationships were thoroughly discussed by speakers who preceded me on this program.

Another antimetabolite which has been used in the study of hemopoiesis is desoxypyridoxine. This has been extensively studied by Dr. Vilter and his colleagues.[10] Certain purine antagonists such as 2,6-diaminopurine have also been found to depress the formation of blood cells.[11]

Prothrombin may be properly regarded as an essential constituent of the blood and its formation is known to be depressed by the antagonists of vitamin K.

Summary (Part I)

(1) Folic acid antagonists are useful in the study of hemopoiesis in experimental animals. (2) Their use in studying blood formation in patients is limited by the toxicity of aminopterin and by the fact that x-methyl folic acid has no apparent effect on human subjects. (3) The use of the folic acid antagonists has established the role of folic acid in blood formation (a) in species which do not readily develop a dietary deficiency of folic acid such as dogs and pigs, (b) in other species which are subject to this dietary deficiency, such as rats and chicks, because the antagonists may be used to exaggerate the deficiency and to establish its reversibility. (4) Studies with the folic acid antagonists in bacteria have illuminated the role of folic acid in biochemistry and hence in hemopoiesis.

II. Antibiotics

More obscure and variable than the action of antimetabolites is the hemopoietic response that certain antibiotics will produce in megaloblastic anemias. The beginnings of the basis for this phenomenon go back to findings which led to the proposal that a component producing or aggravating pernicious anemia may be produced in the gut by intestinal bacteria. More definite evidence in this field came from the observation by Cameron, Witts, and co-workers[12] that an anemia could be produced in rats by segregating an intestinal loop.

About five years ago we noted that antibiotics had a beneficial effect on the growth of young animals which were apparently healthy and which received complete diets. This "antibiotic growth effect" was examined from several standpoints by ourselves and others. It is possible to observe the effect in healthy animals which are on complete diets. A more marked growth effect, however, may often be observed in animals which are suffering from a subacute bacterial infection, especially when diarrhea is evident. A third series of investigations was concerned with the antibiotic growth effect as shown in animals which were subjected to specific vitamin deficiences. We noted, for example, that antibiotics often produced a greater growth response at suboptimal levels of vitamin B_{12} than at optimal.

Observations that dietary antibiotics had a beneficial effect on nutrition came as somewhat of a surprise, in view of the effect of the sulfonamides which were known to depress the synthesis of certain vitamins produced by the intestinal bacteria. These vitamins included some hemopoietic factors, one of which was folic acid. The antibiotics, on the other hand,

were soon found to have a sparing effect on both folic acid and vitamin B_{12} in animals on diets which were restricted with respect to these two vitamins. These considerations led to an investigation of the dietary effects of antibiotics on pernicious anemia by Lichtman and co-workers.[13] Four patients with pernicious anemia in relapse and one patient with nutritional macrocytic anemia responded to the oral administration of Aureomycin,* 2 to 3 Gm. daily. A fifth patient with pernicious anemia failed to respond to 0.6 Gm. of Aureomycin given intravenously daily for 20 days, thus indicating that the Aureomycin was not contaminated with significant amounts of hemopoietic factors such as vitamin B_{12} and folic acid. Microbiological assay of the Aureomycin with *Euglena gracilis* indicated a vitamin B_{12} content not greater than 0.17 μg. per gram, and hence negligible; furthermore, case No. 3 did not respond to vitamin B_{12} alone, 3 μg. orally per day, during a 10-day period of pre-treatment. It seems evident, therefore, that the effects observed by Lichtman *et al.* were not due to the presence of vitamin B_{12} or folic acid in the Aureomycin but were more probably caused by changes in the bacterial flora. The possible effects of such a change include increases in the production of folic acid or vitamin B_{12} in the intestinal tract or an improvement in the uptake of these vitamins. The response of the nutritional megaloblastic anemia case indicates that folic acid was involved.

Along similar lines was a report by Foy and co-workers[14] who found that cases of megaloblastic anemia of pregnancy responded promptly to injections of penicillin. These investigators also found that oral penicillin produced responses in certain cases of megaloblastic anemias, but other cases did not respond. Ungley also reported failures in patients with pernicious anemia to respond to streptomycin and Aureomycin.[15]

Recently Foy and Kondi[16] suggested that the megaloblastic anemias studied by them fall into three types with regard to their responses to penicillin or vitamin B_{12}. The first type, typified by pernicious anemia, did not respond to penicillin or to oral vitamin B_{12} but responded to parenteral vitamin B_{12}. The second type, typified by the megaloblastic anemia of pregnancy, did not respond to penicillin or to vitamin B_{12}, even when given parenterally, but responded to folic acid given orally. The third type responded to penicillin or to oral vitamin B_{12}; presumably a dietary deficiency of vitamin B_{12} existed in these patients. These observations led to the suggestion that penicillin influenced the biosynthesis and utilization of intestinal vitamin B_{12} and that this utilization could take place only in the presence of intrinsic factor. Experiments by ourselves and others have indicated that antibiotics sometimes diminish the dietary requirement for vitamin B_{12}; but in our studies with vitamin B_{12}-deficient chicks we have never observed more than a partial effect of antibiotics in sparing or replacing vitamin B_{12}. The diet which we use for assaying vitamin B_{12} with chicks contains added penicillin.

Most of the evidence indicates that nutritional effects of antibiotics are due to changes which these substances produce in the intestinal flora. Irregularities may therefore be expected in the effects of antibiotics on hemopoiesis.

The question of citrovorum factor synthesis in the intestine was studied by Waisman and co-workers[17] and by Schwarz.[18] Waisman and his group found that Aureomycin counteracted the toxic effects which were produced by adding aminopterin to the diet, which suggested that the antibiotic increased the synthesis of citrovorum factor by the intestinal bacteria. We have made several attempts to confirm the finding reported by Waisman, but in our hands Aureomycin had little or no effect in reversing the toxic effects of aminopterin on rats and mice. Our experiments are summarized in Tables VII, VIII, and IX. Sauberlich has also been unable to reverse aminopterin toxicity with Aureomycin or penicillin.[19]

The effect of dietary Aureomycin on the blood constituents of dairy calves was studied

* The trademark of the American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.

TABLE VII

Effects of Aminopterin and Aureomycin on Rats (Purified diet with sulfasuxidine and without folic acid)

Expt.	Supplements	Weight gain in 9 days	Per cent mortality 9 days	14 days
		Gm.		
1	None	31	0	0
1	2 mg. aminopterin (Am.)	−5	83	100
1	2 mg. Am. + 50 mg. Aureo.	−9	33	83
1	2 mg. Am. + 200 mg. Aureo.	5	67	100
		8 days	8 days	
2	None	27	3	
2	25 μg. aminopterin*	10	80	
2	25 μg. Am. + 50 mg. Aureo.	10	83	

* Injected 3 times weekly.

TABLE VIII

Effects of Aminopterin, Aureomycin, and Citrovorum Factor (CF) on Mice on Purified Diet—Experiment 1

Supplements	Weight gain in 11 days	Per cent mortality 11 days	14 days
	Gm.		
None	5	20	20
100 mg. Aureo.	6	10	10
10 μg. Aminopterin (Am.)*	−4	90	100
10 μg. Am. + 100 mg. Aureo.	0	70	100
10 μg. Am. + 10 μg. CF*	2	0	0

* Injected 3 times weekly.

TABLE IX

Effects of Aminopterin, Aureomycin, and Citrovorum Factor (CF) on Mice on Purified Diet—Experiment 2

Supplements	Weight gain in 11 days	Per cent mortality 11 days	14 days
	Gm.		
None	6	0	0
1 mg. Aminopterin (Am.)	−3	27	73
1 mg. Am. + 20 mg. Aureo.	−3	18	27
1 mg. Am. + 40 mg. Aureo.	−3	36	55
1 mg. Am. + 100 mg. Aureo.	−3	36	82
2 mg. Am.	−4	91	91
2 mg. Am. + 20 mg. Aureo.	−5	91	91
2 mg. Am. + 40 mg. Aureo.	−4	82	91
2 mg. Am. + 100 mg. Aureo.	−6	73	100

by Rusoff and co-workers,[20] who found a slight decrease in the leukocyte counts of the supplemented animals. In a similar study, Owen and Allen[21] reported no effect on the erythrocyte, leukocyte, and differential leukocyte counts of dairy calves receiving various antibiotics. Increases in the hemoglobin level of the blood of pigs were found to accompany the feeding of Aureomycin by Burnside and co-workers.[22] Barnard[23] has studied the effect of antibiotics on a large number of patients with refractory anemias and has obtained encouraging results. He has suggested that the hemopoietic function of the bone marrow may be impaired by products of bacterial metabolism which arise in the gastrointestinal tract. He considers that these products are not produced when suitable antibiotics are administered. A number of investigators have noted the production of macrocytic anemia following instestinal stricture, and a megaloblastic anemia may be produced by the surgical isolation of a loop of small intestine. Watson and Witts[24] found that dietary Aureomycin had a beneficial effect on rats which had developed anemia after the surgical formation of a diverticulum in the small intestine.

It is important in conducting such experiments to be sure that the antibiotic does not contain hemopoietic factors as impurities. Vitamin B_{12} is produced by certain microorganisms which are commonly used in the manufacture of antibiotics. One must also bear in mind the possibility that the administration of antibiotics may change the metabolism of the host so that a hemopoietic response is produced. This might be the case in an anemia associated with infection.

Summary (Part II)

It appears that certain antibiotics may have a beneficial effect on hemopoiesis. This effect is associated with changes which the antibiotics produce in the intestinal bacteria. Among the possible changes are: (1) an increase in the production of substances in the folic acid group including citrovorum factor; (2) certain bacteria may destroy vitamin B_{12} and this destructive effect may be lessened by

administering antibiotics; (3) there is some evidence that the intestinal bacteria may produce deleterious substances which cause anemia, and that the production of these substances is suppressed by antibiotics.

The antianemic effects of antibiotics are irregular, due perhaps to inherent variations in the types and numbers of bacteria in the intestinal tract of the experimental host.

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Stercobilin and Hematopoiesis

By G. Watson James, III, M.D.*

This discussion considers some of the recent investigations, fundamental concepts, biochemical interrelationships, and hypotheses relative to the finding that 10 to 20 per cent of the fecal stercobilin excretion is more intimately associated with erythropoiesis than with erythrocyte destruction. The wisdom and work of many investigators—Shemin, London, Rittenberg, Wittenberg, Neuberger, Gray, Watson, Lowry, and others—has broadened and crystallized new ideas on the association of pigment production and red cell formation.

The term stercobilin is used in preference to urobilin or urobilinogen, since stercobilin is the major final product of intestinal bilirubin degradation in the normal man or in one who has not recently received antibiotics of the tetracycline group. Fortunately the Ehrlich reaction with *p*-dimethylaminobenzaldehyde gives quantitatively the same color complex, and, for purposes of discussion, stercobilinogen, or stercobilin, and urobilinogen are used interchangeably.

Metabolism of Stercobilin

Eighty to 90 per cent of the total fecal and urine stercobilin excretion results from the destruction of circulating erythrocytes. Degradation of the hemoglobin molecule to bilirubin can occur in any reticuloendothelial macrophage of the body, whether this be in the skin, spleen, liver, in cysts, or elsewhere. The exact steps of catabolism are not clearly understood, but in general the molecule is converted from a cyclic tetrapyrrole containing iron and protein to an open chain compound to which the iron and protein are still probably affixed. In subsequent steps in the reticuloendothelial cells and liver cord cells the iron and protein are removed to be reutilized, and the pigment fraction is excreted in the bile as bilirubin and biliverdin. Studies by Hawkins and Whipple[1] in the dog suggested a nearly quantitative relationship between bile pigment excretion and the loss of hemoglobin from the circulation.

Studies in man, however, have left much to be desired because of the discrepancies which have appeared from a comparison of theoretical and actual quantitative results for stercobilinogen excretion. For example, a 70-kilogram man whose total circulating hemoglobin is 875 grams destroys approximately 0.83 per cent per day, or about 7.3 grams, which results in the formation of about 260 milligrams of bilirubin and hence stercobilin per day. Results in normal men studied over a four-month period in our laboratory (Fig. 1) indicate that the measured daily excretion is much less than the calculated excretion. The differences come from imperfect methods for urobilinogen determination, reabsorption and further breakdown in the liver, and degradation in the urine and feces to mono- and dipyrroles not giving the Ehrlich reaction.

The demonstration by four separate groups[2–5] that from 10 to 20 per cent of the fecal stercobilin may come from sources other than the degradation of the circulating erythrocytes is of considerable interest. The key to this significant discovery has been the utilization of the heavy isotope of nitrogen (N^{15}) from labeled glycine to tag the pyrrole ring in the pigment fraction of hemoglobin. London[2] *et al.* gave labeled N^{15} glycine orally to one and intravenously to another young adult and followed the uptake of the nitrogen in the heme of the erythrocytes and the appearance of the nitrogen in the stercobilin of the feces. If stercobilin came solely from the destruction of the circulating erythrocytes,

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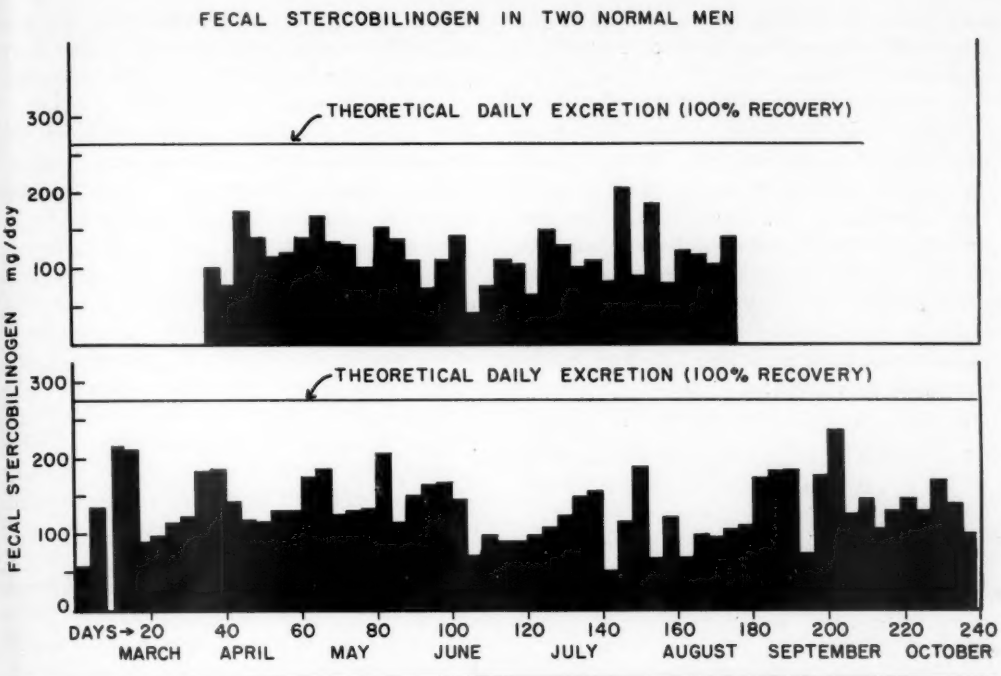

Fig. 1. Fecal stercobilinogen excretion in two normal adult males. There is considerable daily variation, as well as consistent incomplete recovery of the theoretical amount of stercobilinogen formed.

no significant label should have been found in the feces until after the sixtieth to seventieth day, when breakdown of the labeled red cells begins. The startling finding was an initial high label in the stercobilin at the time when the N^{15} concentration of heme in the circulating erythrocytes was small. In this original experiment London[2] *et al.* pointed out that the circulating erythrocytes could not be the source of this initial stercobilin tag, and they calculated that at least 11 per cent of the stercobilin was coming from a non-hemoglobin source.

Recent work[7] in our laboratory in a normal male fed istopic glycine again clearly demonstrates this interesting observation. The subject was fed first in a steady state, then, after thirty days, he was bled to give the labeled cells to a normal recipient. Under the stimulus of this moderate hemorrhage, a reticulocytosis of 4–5 per cent developed, and he was fed an identical amount of N^{15} glycine. Figure 2 shows the results. On the vertical

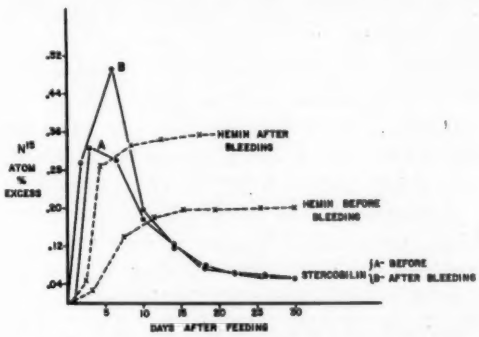

Fig. 2. Appearance of heavy nitrogen in hemin and fecal stercobilin in a normal adult given N^{15}-glycine before and after phlebotomy.

axis, hemin and stercobilin N^{15} are given in atom % excess. Time, in days, is plotted on the horizontal axis. Considering the hemin curves it is clear that a greater concentration was reached in a shorter period of time after the bleeding. At the fifth day after glycine feeding, the concentration of heme N^{15} was 4.3 times greater after bleeding than at the

same period in the normal state. Not only is the uptake greater, but the rate of uptake is considerably faster.

A qualitative comparison of the quantity of stercobilin arising from a non-hemoglobin source in this experiment can be made, using the type of calculation employed by London[2] *et al.* Briefly: it is a logical assumption that concentration in the stercobilin can be estimated from the average concentration in the newly formed heme if they have the same precursor. The concentration in the newly formed heme during the first five days can be calculated as follows:

Let

C_H = average concentration N^{15} in atom % excess entering circulation in the red cells during time "t"

l = average life span of the red cells concerned

c = observed average concentration N^{15} in atom % excess during time "t"

Then:

$$C_H = cl/t$$

or

$$C_H = \frac{0.068 \times 120}{5} = 1.63 \text{ atom \% excess}$$

On the assumption that this approximates the concentration on the precursor of the stercobilin whose observed label at five days was 0.312 atom % excess, it can be calculated that 0.312/1.63 = 0.191, or 19.1 per cent of the stercobilin was coming from non-hemoglobin precursors. When the same type calculations are made after bleeding, it is found that the average concentration in the hemin in the newly formed cells at five days is 7.02 atom % excess. The stercobilin concentration at this time was 0.440 atom % excess so that 0.440/7.02 = 0.063, or 6.3 per cent is coming from non-hemoglobin precursors. This suggests that, in this man, as hemoglobin synthesis became more rapid, he excreted less of the non-hemoglobin fraction of stercobilin.

Source of Pigment

The source of this stercobilin is of considerable interest. There are several possibilities. First, bile pigment may come from the immediate breakdown of red cells which have never left the bone marrow or are destroyed immediately upon reaching the circulation. This may be likened to fish eggs—billions are produced, among which some are defective and immediately destroyed. It is hard to suppose that in man erythropoiesis is so inefficient as to immediately destroy 10 to 20 per cent of the red cells produced in a day. Another explanation may be found in two separate populations of red cells, a short-lived and a long-lived one. There are certain calculated data from C^{14}-isotopic studies in polycythemia vera[8] and N^{15} labeling in sickle cell anemia[9] that suggest this possibility. Yet, it has not been clearly demonstrated in the normal man, and we[10] have been unable to demonstrate it in a patient with severe sickle cell anemia. Thorell[11] recognized in pernicious anemia that the synthesis of blood pigment may proceed without disappearance of ribose polynucleotide in the maturing erythrocyte. It is possible that any excess pigment formed is excreted. London[12,13] demonstrated *in vivo* that both hematin and protoporphyrin could be converted into stercobilin, and thus the complete ring structure of hemoglobin need not be formed. Other sources of pyrrole in the body, such as cytochrome, peroxidase, and catalase, have rapid turnover rates, but the quantities involved are probably too small to contribute such a major fraction of stercobilin. London *et al.*[14] have considered that myoglobin, the next largest source of tetrapyrrole rings, has such a slow turnover rate that it seems unlikely that it could be the source of this initial stercobilin.

Abnormal Metabolism

The study of nutritional, metabolic, or congenital disorders often gives a valuable guide to the pathways of normal physiologic mechanisms. Pernicious anemia and congenital porphyria are two diseases in which significant abnormalities have been demonstrated by the N^{15} techniques in the initial stercobilin fraction.

Vitamin B_{12} deficiency in the human results in a fundamental metabolic disturbance of

erythropoiesis and changes in pigment metabolism which are probably closely associated with the change from normoblastic to megaloblastic erythropoiesis. The high fecal excretion of stercobilin in the untreated patient with pernicious anemia, as well as the known remarkable effect of vitamin B_{12} in reducing this abnormality, should provide some information relative to stercobilin formation in erythropoiesis. The results obtained by London[14] in a study of stercobilin formation in pernicious anemia (Fig. 3) clearly demonstrate a striking abnormality. His patient in

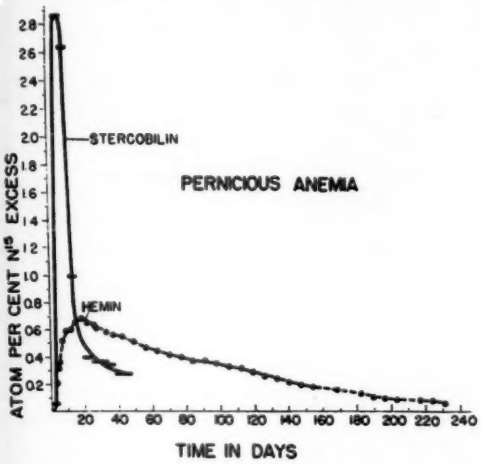

Fig. 3. Initial stercobilin N^{15} content in a patient with pernicious anemia fed oral N^{15}-glycine before treatment (London and West. *J. Biol. Chem.* 184: 359, 1950).

relapse was fed isotopic glycine, and from the initial high label on the stercobilin he calculated that at least 40 per cent of the fecal pigment was derived from a non-hemoglobin source. In our laboratory we[10] have given orally one gram of N^{15}-glycine to a patient with pernicious anemia during the reticulocytosis in vitamin B_{12}-induced remission. The label observed in the stercobilin was much reduced, so that the estimated amount of stercobilin coming from a non-hemoglobin source was less than 10 per cent.

Whatever the abnormal mechanism may be, it is very quickly corrected by the administration of the vitamin B_{12}, the action of which is probably not involved directly with porphyrin ring synthesis, but is more likely associated with nucleoprotein formation and the subsequent intracellular synthesis of protein. Yet, DeMello[15] reports that small amounts of vitamin B_{12} are effective in preventing the coproporphyrinuria produced in rabbits by intravenous administration of Rose Bengal and subsequent photosensitization by ultraviolet light. It is felt that the defect observed in pernicious anemia in which bile pigment excretion is increased could be due to a failure of pigment utilization. Pigment synthesis as such is probably normal in pernicious anemia, but the formation of globin or the time relationship of pigment to protein formation may result in the excretion of non-utilized pigment. Studies similar to those performed in pernicious anemia need to be done in the folic acid deficiency states, where the megaloblastic marrow picture is nearly identical, but where the clinical evidence of excess pigment production is not as striking.

In a patient with congenital prophyria whose erythrocytes appeared to live a normal life span, Gray *et al.*[3] demonstrated a very high initial stercobilin N^{15} label (Fig. 4). He calculated that as much as 85 to 90 per cent of

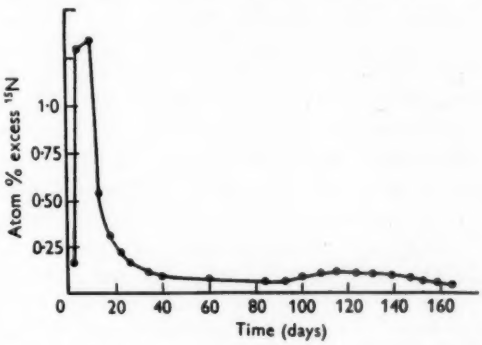

Fig. 4. N^{15} content of stercobilin samples obtained at various times after administration of N^{15}-glycine to a congenital porphyric. (Gray and Sneath. *Biochem. J.* 47: 87, 1950).

the fecal stercobilin was coming from a non-hemoglobin source. This stercobilin was chemically and physically indistinguishable from stercobilin isolated from a normal individual. Gray *et al.*[3] believe, from their studies in the porphyric and in the normal

man, that the initial fraction of stercobilin is associated with the process of red cell formation, and suggested that increased rates of destruction or increased rates of formation would be associated with increased amounts of stercobilin production. Our studies[7] in a normal man, as shown earlier, suggest that with normally increased erythropoiesis, less stercobilin appears to come from a non-hemoglobin source.

London *et al.*[16] calculated in a similar patient with cells of normal life span that 31 per cent of the fecal stercobilin came from a non-hemoglobin source. Grinstein *et al.*[6] were unable to determine the life span of the erythrocytes in the patient with congenital porphyria that they studied, and so could not determine the relative amount of stercobilin coming from a non-hemoglobin source. They thought the initial high stercobilin N^{15} label was due to the immediate destruction of newly formed erythrocytes, and hence the initial stercobilin fraction was catabolic rather than anabolic. The patient had a moderately severe hemolytic anemia.

Stercobilin and Porphyria

The results observed in congenital porphyria suggest that the early fraction may be associated with the biosynthesis of the porphyrin ring. New developments have come in the last year from the laboratories of both the American and English workers. Shemin and Russell[18] have reported that a new intermediate *delta*-aminolevulinic acid could replace both "active" succinate and glycine in the synthesis of protoporphyrin. Using C^{14}-glycine and N^{15}-glycine in an *in vitro* system of duck erythrocytes, they found that the radioactivity of hemin produced from labeled "active" succinate and unlabeled glycine or vice versa was reduced 80 to 90 per cent by the addition of equimolar amounts of unlabeled *delta*-aminolevulinic acid. They postulated that "active" succinate (possibly succinyl coenzyme A) condenses with glycine to form an obligatory intermediate *alpha*-amino-*beta*-ketoadipic acid, which by decarboxylation would give *delta*-aminolevulinic acid. The condensation of two moles of this compound (Fig. 5) would produce a hypothetical pyrrole precursor. These findings have been confirmed in England by Neuberger and Scott.[19] It is particularly significant that the theoretical substance formed in this condensation has the same proposed structural formula as porphobilinogen, which has recently been crystallized by Westall[20] from acute intermittent porphyria (porphyria hepatica) urine, and whose structural formula has been suggested by Cookson and Rimington[21] and by Granick and Bogorad.[22]

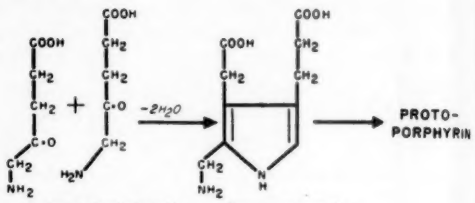

Fig. 5. The formation of the precursor pyrrole for porphyrins from two moles of *delta*-aminolevulinic acid (Shemin and Russell. *J. Am. Chem. Soc.* 75: 4873, 1953).

This monopyrrole might be the precursor of the type III porphyrins in the developing erythrocyte, since red cell and liver hemolysates rather easily convert crystalline porphobilinogen into uroporphyrin, coproporphyrin, protoporphyrin, and other unidentified porphyrins.[23]

Porphobilinogen, however, does not appear in the urine of the congenital porphyric patient, who, in addition to the excretion of large quantities of free porphyrin, often has evidence of mild to moderate hemolytic anemia. This suggests that the monopyrrole precursor can be condensed into ring tetrapyrroles and that the excess pigment formed could be converted in part to stercobilin, or that stercobilin occurs as a normal by-product in erythropoiesis. Porphobilinogen excretion occurs characteristically in most patients with porphyria hepatica of the acute intermittent type. Sufficient N^{15} studies have not yet been performed in this class of porphyria patients, but Lowry and his co-workers[5] found a normal amount of the early stercobilin fraction in a patient with "mixed" porphyria, another sub-

type of the porphyria hepatica classification. In porphyria hepatica, the major defect in porphyrin metabolism appears to occur in the liver.

It is perhaps significant that the peak of the initial stercobilin fraction comes at about the same time that the peak N^{15} label occurs in the fecal coproporphyrin, as found in congenital porphyria studies of London *et al.*[16] and Gray *et al.*[3] When our data for the initial stercobilin fraction times the necessary multiplication factor are plotted on the same co-ordinates (Fig. 6) with the data of Gray *et al.*,[3] the time of the greatest tag on the fecal stercobilin is about the same as that

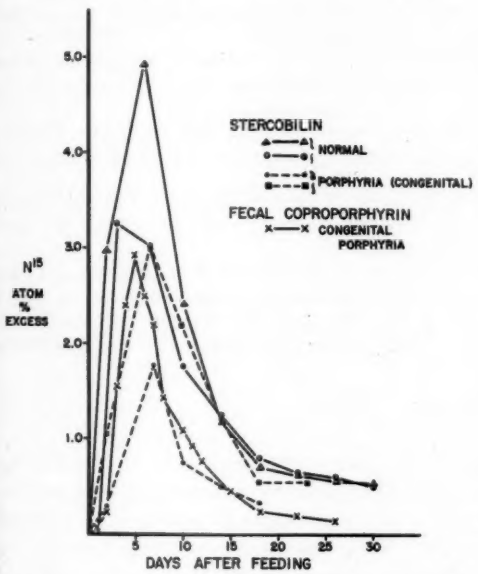

Fig. 6. Stercobilin N^{15} data and fecal coproporphyrin N^{15} data from the normal and porphyric plotted together to show similar time relationships. The porphyrin data are from Gray.[3]

found in the fecal coproporphyrin, and the slope of descent is about the same. Grinstein *et al.*[6] did not find this in their patient and thought the fecal coproporphyrin isotope decline was faster than the stercobilin isotope decline. Both these disappearances probably reflect the disappearance of the N^{15}-glycine precursor. The initial stercobilin formation must occur very rapidly, since the highest concentration of N^{15} is found in the first four or first eight days after oral administration.

The arrangement of the side chains of bilirubin is similar to that of protoporphyrin. We may speculate that a linear tetrapyrrole might be formed which goes directly to bilirubin without ring closure. This substance might be an important intermediate whose direction of ring closure or non-ring closure is dependent upon a process which is disturbed in pernicious anemia, but corrected in some unknown manner by vitamin B_{12}, and overwhelmed in porphyria erythropoietica because of the fundamental disturbance in this disorder in bone marrow pigment metabolism. Apparently, however, this process remains normal in porphyria hepatica, which is not a fundamental disturbance of erythropoiesis.[17]

Summary

Evidence gathered from the work of many investigators shows that 10 to 20 per cent of the fecal stercobilin excretion is closely related to erythrocyte and hemoglobin formation, rather than to erythrocyte destruction. This fraction is reduced when erythropoiesis is stimulated by hemorrhage in the normal man. The fraction is markedly increased in porphyria erythropoietica, but not in acute intermittent porphyria; it is also increased in pernicious anemia, but corrected in the latter by vitamin B_{12} therapy. The source of this stercobilin is not known, but it is perhaps related to the biosynthesis of the porphyrin ring from monopyrrole precursors.

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DISCUSSION

Dr. A. D. Welch (New Haven, Conn.): I might add something to what Dr. Jukes commented on concerning the over-production of citrovorum factor, which is thought to be involved in making the cell resistant to aminopterin. Nichol, in our laboratories, has studied this phenomenon and we are rather doubtful at the time as to whether this hyperactivity, if you will, with respect to producing citrovorum factor, is actually an essential part of the mechanism of resistance. It is an extremely interesting phenomenon but I rather suspect it is the result of a separation alteration; perhaps there is a double selection involved—of properties acquired through separate mutations. In any case, the aminopterin-resistant cell can be shown to have within it the enzyme system concerned with the conversion of pteroylglutamic acid to citrovorum factor and this enzyme system is just as sensitive to the analogue as is that in the parent cells. This was shown by preparation of a cell-free extract by ultrasonic vibration and tests of the susceptibility of the extract of the resistant cells to inhibition by the antagonist. The presence of inhibitor-sensitive enzyme within resistant cells would seem to indicate that these cells had developed a barrier to the entrance of the analogue or in some way had lost the capacity to let it in. At any rate, the enzyme was there and it was just as sensitive as was that of the inhibitor-sensitive cells; yet, in the intact inhibitor-resistant cells it was not inhibited.

Another development which I might mention that Dr. Nichol will report on shortly re-

lates to the fact that the structure of the citrovorum factor derivative from which citrovorum factor as such is obtained is not yet known. It appears that in the isolation of the citrovorum factor from natural materials this material is cleaved from a complex labile derivative. The citrovorum factor *per se*, as now formulated, is not the active portion of the enzyme; rather, it appears to represent a degradation product of a co-enzyme.

DR. T. H. JUKES (Pearl River, N. Y.): You wouldn't suggest, would you, Dr. Welch, that the actual factor differs in the essential points?

DR. WELCH: No.

DR. JUKES: We may still assume that the reduced pteridine ring is present in the formyl group.

DR. C. D. MAY (Iowa City, Iowa): I can't let this opportunity pass and hope to precipitate some comment from Dr. Jukes or Dr. Welch, or others, to guide us in our evaluation of the use of antagonists as a tool in studying human nutrition; that is, the advantages are obviously great. I have in mind, for example, the treatment of the leukemic child with aminopterin when one sees signs of apparent toxicity. Sometimes these are not features of folic acid antagonism as we usually understand them.

I would appreciate some guidance in terms of our use of these tools and the evaluation of the results.

DR. JUKES: The oral fissuring we observed in rats was preventable by folic acid, so I feel that the picture which is produced by these highly toxic substances is nevertheless a highly intensified, generalized, and more extensive picture of folic acid deficiency than can be readily obtained by dietary means. I think that the most important criterion of whether or not the symptomatology is truly a folic acid deficiency is whether it is reversible by citrovorum factor.

Essential Nutrients *in the Management of* Hematopoietic Disorders of Human Beings: *A Résumé*

By Richard W. Vilter, M.D.*

It is probable that all nutrients are necessary for the formation of the normal erythrocyte, leukocyte, and platelet, and of the plasma in which these elements float. Most of these substances are so readily available that they are never limiting factors in blood formation. A select few are not so accessible to the bone marrow, cannot be synthesized by the body, and, when a deficiency occurs, may limit blood formation. These are the essential nutrients around which this conference has revolved. These are iron, for hemoglobin formation, and folic acid and vitamin B_{12}, for nucleic acid synthesis. It is probable that protein and particularly some of the amino acids are just as important and just as likely to be deficient when diets are inadequate or stress excessive. However, so little is known concerning the protein requirements for hematopoiesis in human beings, that no full-dress discussion of the subject was considered to be profitable. There has been clear demonstration that the protein requirements for hemoglobin formation in anemic hypoproteinemic dogs takes precedence over serum protein and tissue protein formation. In hypoproteinemic animals with normal hemoglobin levels, hemoglobin and tissue protein have equal priority. In animals, protein and tryptophane, methionine, histidine, phenylalanine, and lysine deficiencies induce an anemia which is usually microcytic and slightly hypochromic. Our group here in Cincinnati has been impressed with a form of nutritional hypoplastic anemia in exceedingly malnourished persons. We have thought that this anemia might be the result of protein malnutrition, but have not been able to demonstrate a response to casein supplements alone, although these persons will recover slowly when fed a complete diet. In kwashiorkor, a disease due primarily to protein malnutrition, no specific type of anemia has been described. Most infants with this syndrome are anemic, but the cause is usually iron deficiency, chronic infection, or parasitic infestation. Even though we have not established protein as a limiting factor in blood formation in human beings, the basic need for protein in blood regeneration is recognized and a diet rich in animal and vegetable protein should be a basic tool for the treatment of anemia. If it does nothing else, it may keep hemoglobin regeneration from stealing protein from some other part of the body.

Likewise, much time could have been spent on a discussion of the niacin, riboflavin, and vitamin B_6 requirements of laboratory animals for blood formation. These vitamins, the precursors of important oxidation, decarboxylation and transamination enzyme systems are essential parts of every growing cell; yet in human beings, deficiencies of these substances have not been shown to induce anemia. Apparently, other organs and systems are so adversely affected that death occurs before the function of the bone marrow is seriously impaired. Nevertheless, since we recognize their importance to all cellular metabolic functions, we should prescribe a diet rich in the B-complex vitamins when we treat nutritional anemia. Until a patient can take a diet rich in protein and these vitamins, we may

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wish to supplement his intake with protein in the form of powdered milk and with a mixed-vitamin capsule. We must recognize that this prescription is merely in lieu of an adequate diet and has no specific effect on blood formation. It is supportive therapy.

Bile pigment is a very important part of the hemoglobin molecule, but there is no evidence that it ever becomes a factor limiting blood formation. Rather, as Dr. James has shown, it is important as a means of studying blood destruction and some of the steps in the synthesis of porphyrins and hemoglobin. Of all the essential nutrients, only folic acid and vitamin B_6 might be limiting factors in pigment metabolism, but, as yet, we know of no therapeutic considerations in this field.

As has been pointed out many times, iron, folic acid, and vitamin B_{12} have very specific metabolic functions. Ascorbic acid influences the metabolism of these substances, too. These functions are so specific, in fact, that accurate diagnosis of the type of anemia is essential; otherwise, nine times out of ten the therapy will fail, and the patient's money and time will be wasted. On the other hand, if diagnosis is accurate, a deficiency of one of these substances is found to exist and if the physician sets about correcting it by giving the deficient substance by a satisfactory route of administration, a brilliant therapeutic result will be obtained.

Dr. Carl Moore has shown us that the absorption of food iron is improved by vitamin C or other reducing agents. Inorganic iron as used in treatment—ferrous sulfate and ferrous gluconate, given in full doses of one or two grams daily—does not require accessory substances for satisfactory absorption or utilization. A ferrous sulfate or ferrous gluconate tablet costs a penny, and delivers to the patient just as much, if not more, iron than a complex formula containing many nutrients essential for other things but not for blood formation in the iron-deficient patient. For those persons whose irritable gastrointestinal tracts are upset by the simple iron compound—and one finds these in the carriage trade practice much more frequently than in the free or part pay clinic—one may reduce the dose or give saccharated oxide of iron by vein or mouth.

Two warnings should be given at this point:

1. Be sure you know the source of blood loss in the patient with microcytic hypochromic anemia, and deal with it as effectively as possible.

2. Don't use iron as a placebo—you may be giving it to a patient with incipient hemochromatosis.

Dr. George Cartwright has presented a beautiful physiologic study of copper in hematopoiesis and has demonstrated that this element is necessary for the absorption, utilization, and movement of iron from iron stores. He has also shown us that the anemia of copper deficiency in swine is due, at least in part, to the formation of an erythrocyte whose life span is short. There is still more to be learned concerning copper in hematopoiesis, but there is no need for this element in pill or capsule form except under the rarest of circumstances. It is difficult to find a diet containing an insufficient amount of copper; a new-born infant has large copper stores even though his blood plasma copper level is low; and inorganic iron preparations contain sufficient copper as an impurity.

The functions of cobalt in hematopoiesis are unknown, except as this element is an essential part of the vitamin B_{12} molecule. Cobalt, in large doses, 100 to 150 mg. daily as cobalt chloride, will induce polycythemia in human beings. It will force the bone marrow to make more cells even when nephritis or chronic infection are the causes of the anemia. It is doubtful whether this effect is physiological, and the benefits of such therapy are still questioned by most hematologists.

The other trace elements, zinc, molybdenum, magnesium, and manganese have not been shown to effect hematopoiesis.

Much has been learned in recent years of the metabolism and clinical interrelationships of vitamin B_{12}, folic acid, and ascorbic acid. Doctors Williams, Mueller, Will, and Schilling have covered this field in detail, and Dr. Jukes has shown how antimetabolites of some of these substances have been used successfully in hematologic research. These hematopoietic

vitamins are very specific chemical compounds with clear-cut metabolic functions. These are: the formation of the purine ring, the interconversion of pyrimidine ribosides, the formation and metabolism of certain essential amino acids, and the maintenance of reduction potentials within the cell. They will improve hematopoiesis only when a deficiency of one of them has interfered with blood formation. Vitamin B_{12}, given parenterally in doses of 10–15 μg. daily, is the therapy of choice in pernicious anemia in relapse and in nutritional macrocytic anemia. Actually, 1 μg. daily is all that is required, but we usually give ourselves a tenfold margin of safety. In larger doses, vitamin B_{12} is usually effective in sprue, but relapse may occur after several years of treatment. It may be effective also in tropical macrocytic anemia and in the anemia associated with blind intestinal pouches or fistulae. It is usually not effective in megaloblastic anemia of infancy, pernicious anemia of pregnancy, and achrestic anemia. In these three conditions, and in sprue, the drug of choice is folic acid administered orally in doses of 5 mg. three times daily. Less is required in the infant, of course. Folic acid and folinic acid are roughly equivalent in these anemias, although the synthetic folinic acid, because of its spatial configuration, is only half as potent as natural folinic acid or folic acid.

Folic acid alone is dangerous to the patient with pernicious anemia, whether it be prescribed by a physician or purchased in a mixed vitamin capsule. It allows cord damage to occur, usually without signs of anemia to give a warning. However, only one microgram of vitamin B_{12} daily is necessary to relieve the neurologic degeneration that usually occurs in patients with pernicious anemia treated with folic acid or folinic acid alone, and such small doses would probably prevent the degenerative changes.

Vitamin B_{12} combined with intrinsic factor for oral use is not equally effective in all patients with pernicious anemia; it is expensive (about 40 cents daily), and cannot yet be compared favorably with 20–30 μg. of vitamin B_{12} given parenterally every three to four weeks (about 40 cents a month) for maintenance of patients with pernicious anemia.

The use of massive doses of vitamin B_{12} in various neuritides and in tic douloureux is not yet supported by undisputed experimental data, though I strongly suspect that where there is so much smoke, there must be some fire. There is clear-cut evidence that vitamin B_{12} is involved in ribose nucleic acid metabolism of nerve cells (Nissl substance), and it may act as a protecting agent under certain circumstances.

Dr. Crafts has shown in rats that the cortical steroids, thyroxine and androgens are essential for normal hematopoiesis and affect the utilization of certain essential nutrients, particularly protein. These hormones probably govern the pace or speed of blood formation by the bone marrow. They are all interrelated, but only ACTH and the cortical steroids will stimulate the bone marrow to increased activity, whether or not there has been a deficiency of the hormone. These same statements probably apply to human beings, too.

I hope all of our guests have enjoyed this Symposium as much as I have. It has been a great pleasure to participate in it. To any investigator whose work has been neglected, I offer my apologies. We have tried to cover a large field in a very short time and, unfortunately, all important subjects could not be mentioned. However, I think this group of speakers has given a clear description of the mechanisms of blood formation known at this time. I hope everyone can see as clearly as I one great lesson for every practicing physician. Each of these nutrients will usually do only one job, and in order to discover that job, an accurate diagnosis must be made. Otherwise we waste money, time, and effort.

Dietotherapy

Infant Nutrition

By LEE FORREST HILL, M.D.*

HUMAN MILK is regarded almost unanimously as being superior to any other type of milk for infant nutrition, and yet there is little question but that the incidence of breast-feeding in the United States is steadily declining. It seems regrettable that this product, specifically designed by nature for the human infant, should be so lightly cast aside by so many. Clearly the explanation lies in the fact that artificial feeding has become so simple, safe, and uniformly successful that breast-feeding no longer seems worth the bother. Numerous articles have appeared in the literature over the years citing the advantages of human milk over cow's milk for feeding infants in the first few months of life, but this approach has obviously had no effect on reversing the trend in the incidence of breast-feeding. In the development of infant feeding in the past half century, artificial feeding has received tremendous attention, whereas breast-feeding has received relatively little. If breast-feeding in the future is to have any chance of competing in popularity with bottle feeding, some different approach than has been attempted thus far seems indicated. Logically, it would seem that the time has come when research efforts should be directed toward the problems of breast-feeding, and wide publicity given to their solution.

BREAST-FEEDING

Casual inquiry into the causes for the progressive decline in the incidence of breast-feeding reveals several of major significance. Foremost among these is a lack of hospital personnel really interested in promoting breast-feeding. With the exception of a few centers where there is an interested personnel, breast-feeding is largely restricted to those women who are so psychologically and physiologically constituted for nursing that they attain success whether or not they receive professional advice and assistance. The many others who start out so hopefully abandon the task after a few days or weeks, usually because some minor difficulty is encountered which could have been anticipated and prevented. Unwillingness on the part of American women to nurse their infants is not, in my opinion, a prominent factor in the declining incidence of breast-feeding. Most primiparae—perhaps four out of five—with a little encouragement express a desire to breast-feed, only to be defeated in the first few days or weeks under the system of neonatal care which prevails in a majority of our hospitals today.

A second factor influencing adversely the incidence of breast-feeding is the current custom of discharging mothers from the hospital on the fifth post-partum day. Rarely have the problems of breast-feeding been resolved by this time, with the result that their further solution becomes the responsibility of the mother at home. Resumption of her household duties plus concern over her infant's welfare are not conducive to milk secretion. The all too frequent result, often on professional advice, is to abandon the breast in favor of the bottle.

The techniques necessary for successful nursing have been demonstrated repeatedly,

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first by Sedgewick[1] in Minneapolis in the 1920's, later in England by Waller,[2] and currently in this country by the Yale Rooming-In Project.[3] The first requirement is preparation for nursing during the latter months of pregnancy. During the last two months, nipples should be toughened by some simple process such as massaging with a rough towel, and colostrum should be expressed manually once or twice a day. Retracted nipples should receive special attention. Waller advises a particular type of breast shield. Post-partum, every precaution should be taken to avoid sore nipples, since this is one of the common causes of failure of lactation. Painful engorgement, untreated, may result in cessation of milk secretion within a few days. Softening of the breast by preliminary removal of milk by gentle manual expression or with the breast pump permits the baby's nursing to be effective, and maintains the secretory function. Obviously, such simple measures as these require the sympathetic advice and assistance of both physician and nursing staffs. On the extent to which they are available and functioning depends in many instances the outcome of lactation. In Waller's hospital, 77 per cent of primiparae nursed their babies for six months. It is to be hoped that in the not too distant future, some group or organization in this country will be stimulated to undertake an intensive nationwide educational campaign designed to promote a progressive increase in the utilization of human milk for infant nutrition.

Human vs. Cow's Milk

Such a plea obviously encompasses the belief that human milk possesses distinct advantages over cow's milk. Some will not agree with this thesis. It must be admitted that as scientific advances in artificial feeding have progressed, the arguments favoring breast-feeding have become fewer and fewer. Indeed, there are many who feel that about the only remaining advantage is a psychological one. The older idea that breast-fed babies could be distinguished from artificially fed babies on the basis of superior nutrition no longer seems tenable. Artificially fed infants on properly constructed formulas thrive and perform equally with infants receiving human milk. For many years, the strongest argument favoring human milk was that morbidity and mortality rates from infections of the respiratory and gastrointestinal tracts were distinctly less among the breast-fed. Undoubtedly this is still true in the poorer sections of the country where facilities for the making and storing of artificial feedings are inadequate, and where professional supervision is lacking. But in those areas where economic standards are average or better, it would appear that advances which have been made in artificial feeding, together with the influence which the chemotherapeutic and antibiotic agents exert upon the control of infections, have greatly lessened or abolished altogether any differences in illness and death rates between the two groups.

Still another belief generally held until recently was that the protein of breast milk was superior nutritionally to that of cow's milk. Casein contributes about one-third of the total protein in human milk, and about 85 per cent in cow's milk. Lactalbumin with a small amount of lactoglobulin makes up the remainder for each milk. Casein was considered nutritionally inferior to lactalbumin, based on the observation that fur-bearing animals thrived better on lactalbumin than on casein. Lactalbumin contains slightly more of the sulfur amino acids, cystine and methonine, than does casein. This difference is of importance to the nutrition of the fur in animals, but obviously is not a factor in humans. Analyses of casein and lactalbumin have failed to show any deficiency in the essential amino acids in casein, and metabolic studies have been carried out in humans which indicate that the two proteins are of equal biologic value.

Also it was formerly held that the fat of human milk was less likely to result in fat intolerance than the fat of cow's milk. One basis for this belief was that the fat globules of human milk were smaller, and therefore more readily digested, than those of cow's milk. However, it was later demonstrated that the fat globules of cow's milk could not

be distinguished from those contained in human milk by microscopic examination of single specimens.

In spite of the difficulty in citing clear-cut evidence supporting the nutritional superiority of human milk over cow's milk, most clinicians with wide experience in infant feeding would agree that, by-and-large, breast-fed infants in the early weeks of life run a smoother course than do their bottle-fed brothers and sisters. It may be, as suggested by Jeans,[4] that human milk contains other unknown factors which make it nutritionally superior to cow's milk. "The basis of the conceded superiority of human milk seems still to be something of a nutritional mystery." [5]

In infant feeding, cow's milk, modified in a great variety of ways, is the usual substitute when human milk is not available. As of today, the physician has a wide selection of simple methods of formula construction from which to choose, any one of which in all probability will be quite staisfactory. He may make his own modifications, using milk in the fresh, evaporated, or dried state, or he may prefer to use one of the proprietary infant foods. Among the latter in most common use are the so-called "one-formula" preparations. These are in powder or liquid form and require only the addition of water to make them ready for infant consumption. Most, when reconstituted, have the same number of calories per ounce (20) as human or cow's milk. The percentage composition of fat, carbohydrate, and protein varies among them, and some have the cow's milk fat replaced wholly or in part by vegetable or animal fats. Additional vitamins are incorporated in the food, and some contain added minerals such as iron. A description of the composition of each preparation is carried on the can. These one-formula foods are designed to be fed unchanged after the neonatal period; the only variation is in the amount given. Such foods are somewhat more expensive than formulas prepared from evaporated or fresh cow's milk, but convenience of preparation is a compensating factor in their favor.

Nutritional Requirements

Whatever the food selected, certain essential requirements must be met if uniform success is to be attained. These were first set forth by Marriott[6] some twenty years ago, and are as applicable today as when first enumerated by him. They are as follows:

1. Sufficient calories.
2. Sufficient protein, carbohydrate, fat, mineral salts, water, and vitamins.
3. Absence of harmful bacteria.
4. Food readily digestible.

The usual caloric requirement for infants is 45–55/lb./day or 100–120/Kg./day. Also, it is generally accepted that a caloric distribution which provides for 15 per cent of the calories from protein, 35 per cent from fat, and 50 per cent from carbohydrate is nutritionally sound for all ages. It is of some interest to compare these percentages with those obtaining in human milk and in cow's milk. For human milk, the percentage distribution is 7 per cent from protein, 42 per cent from carbohydrate, and 51 per cent from fat. For cow's milk, the values are 20 per cent from protein, 30 per cent from carbohydrate, and 50 per cent from fat.

The National Research Council's[7] present recommendation for protein allowance is 3.5 Gm./Kg./day for artificially fed infants. Breast-fed infants who consume 2 to $2^1/_2$ ounces of milk/lb./day receive from 2 to $2^1/_2$ Gm. of protein/Kg./day. Currently, there is some question as to the need for the higher requirement for the bottle-fed infant. A word of explanation on the point may not be amiss. First, there has been the change in the concept that the protein of human milk (predominantly lactalbumin) was nutritionally superior to the protein of cow's milk (predominantly casein). Second, Brennemann's studies showed the importance of the state of the curd in the digestibility and utilization of milk. Considerable nutrient material is lost from the intestinal tract when raw or pasteurized milk is fed, due to indigestible casein curds. By boiling, diluting, drying, evaporating, or acidifying milk, curd tension can be reduced approximately to that of human milk. When one or

more of these processing techniques is employed, as is customary today, utilization of protein from cow's milk may be as efficient as that from human milk. However, the National Research Council states that, "Adequate data are not yet available on the performance of artificially fed infants receiving protein levels comparable to those obtained in breast-feeding." For the present, the previous recommendation of 3.5 Gm./Kg./day remains unchanged, even though it may be somewhat overly generous.

Carbohydrate and the Kidney

A further problem is encountered when the question of adding carbohydrate to the formula is considered. While suggested amounts vary widely with different authorities, it is generally held advisable to add sufficient carbohydrate to bring the total percentage up at least to that in human milk. Carbohydrate is an important source of energy and if insufficient amounts are present in the diet, protein conversion occurs to make up the deficiency. There is yet another important reason for having adequate carbohydrate representation in the formula, as was brought out in a recent study by Pratt and Snyderman.[8] It had been previously contended that infants could be successfully nourished without carbohydrate additions. Pratt and Snyderman fed six rapidly growing small infants on a food mixture of evaporated milk and water only, and compared the renal solute load when the same infants were fed an isocaloric feeding of evaporated milk and water but with added carbohydrate. The renal solute load with the unmodified milk mixture was 85 per cent greater than for the mixture with carbohydrate added. Obligatory water required for the renal excretion of this extra load, while adequate under normal conditions, might well become rather quickly depleted under uncompensated water loss from profuse sweating, vomiting, diarrhea, or impairment of urinary concentration. It is interesting to note that the renal solute load from human milk would be only about one-third of that of the evaporated milk-water-carbohydrate feeding employed by the authors in the above study. Clearly the breast-fed baby has an advantage over the bottle-fed baby in respect to his water reserve.

Unless there are specific indications for varying its amount, such as in the feeding of the premature infant, in some types of vomiting, and in steatorrhea, fat should regularly be represented in the usual formula to the extent that it naturally occurs in milk. It is important as a concentrated source of energy, for its protein-sparing effect, and as the purveyor of the fat-soluble vitamins A and D. As was previously noted, the idea once held that the fat of cow's milk was more indigestible than the fat of human milk is now being questioned. Differences in assimilability, it is maintained, are of minor significance only.

The water allowance in constructing formulas for artificially fed infants requires consideration. Human milk and cow's milk contain approximately the same amounts of water. The breast-fed infant receiving 2 to $2^1/_2$ oz. of milk/lb./day has a caloric intake of 90 to 110/Kg./day and a water intake of approximately 130 to 165 ml./Kg./day. This amount of water from the breast milk itself appears to be adequate for the baby's usual needs. Clinically, this is borne out by the fact that most breast-fed babies refuse water from other sources. Excess losses as from fever, vomiting, diarrhea, or sweating would, of course, increase the requirement. The allowance of 130 to 165 ml. of water/Kg./day is generally regarded as affording a safe margin for both breast- and artificially fed infants. However, as previously indicated, it must be kept in mind that cow's milk imposes a greater osmolar load due to its higher ash and protein content than does breast milk, and hence increases the water requirement for renal excretion. A customary procedure in artificial feeding is to dilute the required amount of cow's milk with sufficient water, after carbohydrate has been added, to make the final formula isocaloric with human milk. Thus, 100 ml./Kg./day of cow's milk ($1^1/_2$ oz./lb./day) supplies 70 cal./Kg./day. Adding 10 grams of carbohydrate raises the calories to 110/Kg./day and adding 65 ml./Kg./day of

water brings the total to the 165 ml. of water/Kg./day and 110 cal./Kg./day, which compares to the breast-fed infant receiving $2^1/_2$ oz. of human milk per pound per day.

Minerals

The total ash or mineral content of cow's milk is over three times that of human milk. Under ordinary circumstances, with the exception of the neonatal period, this excess load of minerals in cow's milk poses no particular hazard for the infant—the excess being rapidly excreted in the urine, at the expense, however, of some of the infant's water reserve.

But in the neonatal period, a hazard does exist in the form of neonatal tetany resulting from the larger content of phosphorus and calcium in cow's milk than is present in human milk, and the less favorable ratio between them. Cow's milk has three times more calcium than human milk and six times more phosphorus. The ratio of Ca:P for cow's milk is 1.3:1 and for human milk 2.2:1. When the formula in the newborn period is too concentrated, the serum phosphorus level becomes elevated. Because of renal immaturity and temporary hypoparathyroidism, the phosphorus remains elevated and calcium decreases. Gardner, Maclachlan, Pick, Terry, and Butler[9] have pointed out the potential danger to the infant in the neonatal period from feeding concentrated cow's milk mixtures. They state that formulas, even diluted two to one with water, may lead to increased serum phosphorus and decreased serum calcium—hence to possible tetany of the newborn. Their recommendation for the artificially fed infant in the neonatal period is a formula composed of cow's milk one part, water two parts, 10 per cent carbohydrate, and added calcium gluconate to produce a Ca:P ratio approaching that of human milk.

The Formula

With the preceding observations in mind, the problem of formula construction may now be approached. A simple method is to construct a standard formula isocaloric with human milk which can be fed unchanged but in varying amounts from the end of the neonatal period to five or six months of age, when whole milk can be substituted. Such a formula should have 20 calories to the ounce, furnish 3.5 grams of protein per kilogram of body weight, have a caloric distribution of 15 per cent from protein, 35 per cent from fat, and 50 per cent from carbohydrate, and supply from 130 to 165 ml. of water per kilogram per day. The following formula closely approximates these requirements:

TABLE I

Standard Formula Using Evaporated Milk (20 cal./oz.)

	Oz.	Total calories	Cal. per oz.
Evaporated milk	11	484	—
Water	22	—	—
Carbohydrate	$1^1/_2$	180	—
		664	20

The percentage composition is approximately 2.3 per cent protein, 2.6 per cent fat, and 7.7 per cent carbohydrate, with a caloric distribution of 14.5 per cent from protein, 37 per cent from fat, and 48.5 per cent from carbohydrate. The amount of the formula an infant would need to consume to meet his total caloric requirement is determined by dividing his caloric requirement by 20, the number of calories in each ounce of the mixture. For example, an infant weighing 10 pounds is to be given 50 calories per pound, or a total of 500 calories per day; 500 divided by 20 equals 25, the number of ounces required. If the entire amount is consumed, the protein intake would be 1.7 Gm./lb./day or 3.7/Gm./Kg./day. The water intake would be $2^1/_2$ oz./lb./day or 165 ml./Kg./day, which would provide adequately for maintenance water under normal conditions. The total quantity of formula has been purposely set at approximately a quart, since it is generally held that this is the maximum amount of milk an infant should receive a day. Solid foods should be used to meet appetite demands beyond this amount.

If it is desired to use fresh cow's milk in place of evaporated milk for the standard formula, construction would be as follows:

TABLE II

Standard Formula Using Whole Cow's Milk

	Oz.	Total calories	Cal. per oz.
Cow's milk	24	480	—
Water	9	—	—
Carbohydrate	$1^1/_2$	180	—
		660	20

A second method of formula construction commonly employed is to provide 1 oz. of evaporated milk or $1^1/_2$ to 2 oz. of fresh cow's milk for each pound of body weight per day. Water is added to allow 2 to $2^1/_2$ oz./lb./day, and sufficient carbohydrate to make the final formula approximate 20 calories to the ounce. An example of this method for a 10-pound infant using evaporated milk is as follows:

TABLE III

Standard Formula Using Weight of Child as Basis

	Oz.	Total calories	Cal. per oz.	Cal. per lb.
Evaporated milk	10	440	—	—
Water	15	—	—	—
Carbohydrate	1	120	—	—
		560	22	56

If fresh cow's milk is used for the same weight infant, formula construction would be as shown below:

	$1^1/_2$ oz./lb.	2 oz./lb.	Total calories	Cal. per oz.	Cal. per lb.
	Oz.	*Oz.*			
Cow's milk	15	20	300–400	—	—
Water	10	5	—	—	—
Carbohydrate	$1^1/_2$	1	180–120	—	—
			480–520	19–21	48–52

Most infants will be able to tolerate the standard formulas described above by three weeks to a month of age. By this time renal and parathyroid functions should be sufficiently mature to permit normal regulation of calcium and phosphorus serum levels. For the reasons previously discussed, the formula for the neonatal period should be less concentrated than the standard formula. According to Gardner and associates, a suitable formula for the neonatal period would be as follows:

TABLE IV

Less Concentrated Formula for Neonatal Period

	Oz.	Total calories	Cal. per oz.
Cow's milk	7	140	—
Water	14	—	—
Carbohydrate	1.0	120	—
		260	13
Evaporated milk	3.5	154	—
Water	17.5	—	—
Carbohydrate	1.0	120	—
		274	13

A gradual transition should be made from the 13 calories per ounce mixture to the 20 calories per ounce mixture of the standard formula as the neonatal age progresses.

"Demand" Feeding

At this point it would seem desirable to interject a few practical considerations about infant feeding. While it is necessary to base formula construction on caloric requirements, in practice it is unwise to force upon the infant, or limit his intake to, amounts of formula that exactly meet his caloric needs. Rather he should be granted the privilege of nursing from the bottle until he is satisfied, in the same manner as when he nurses at the breast. Rapidly growing active infants will obviously consume more calories than those of slower growth and more placid disposition. The principle of self-regulation, both as to quantity and reasonable intervals between feedings, is far preferable to the former rigid schedules. So long as weight gains are adequate, it may be assumed that caloric intake is sufficient. A caloric check may be helpful when the weight gain is considered inadequate.

Frequent formula changes should be avoided. Unless clear-cut indications of indigestion are present, there would appear to be no more reason for changing a properly constructed formula than to change breast milk, if that were possible. The usual reason for changing a formula is because the baby cries or has what is assumed to be "colic." Most often this is not the situation, but merely reflects parental inexperience in managing the baby. Formula changes are infrequent among hospitalized infants where experienced nurses are in attendance.

Vitamins

Finally there remain for consideration vitamin and solid food supplements to the milk diet.

Vitamins A and D

The daily allowance of vitamin A for infants recommended by the Food and Nutrition Board of the National Research Council is 1500 (I.U.) units: a liter of cow's milk contains approximately this amount and human milk considerably more (2300); hence it is apparent that each milk alone supplies the requirement, providing the maternal diet is adequate. However, formula dilutions reduce the vitamin A intake of the artificially fed baby in the first few months, but it is soon restored with the addition of supplemental foods such as egg yolk. While the need for additional vitamin A may be unnecessary, most multiple vitamin preparations routinely used contain it and, unless given in toxic doses, it does no harm.

The recommended allowance for vitamin D is 400 (I.U.) units daily. Both human and cow's milk have negligible quantities of this vitamin. Addition of at least 400 units of vitamin D should, therefore, be begun early in the infant's life, preferably by one to two weeks of age.

Vitamin C

The recommended daily allowance of ascorbic acid is 30 mg. Human milk, if the maternal diet is adequate, contains on the average 43 mg. per liter, whereas cow's milk contains only about 16 mg. per liter. Boiling, pasteurization, or evaporation reduces vitamin C by half or more. The breast-fed baby, providing its mother's diet includes ample vitamin C, has its ascorbic acid requirement met. But artificially fed infants should receive supplements of 30 to 50 mg., beginning shortly after birth.

Vitamins of the B Complex

Vitamins in this category of chief concern in the nutrition of infants are thiamine (B_1), riboflavin, niacin, pyridoxine (B_6) and vitamin B_{12}. The recommended daily allowance of thiamine is 0.3 mg. for the second, third, and fourth months of life, 0.4 mg. for the next six months, and 0.5 mg. for the last two months of the first year. Specific recommendations are not made for the first month of life. Human milk contains approximately 0.16 mg. of thiamine per liter, but there are wide variations. Raw cow's milk contains approximately 0.42 mg. per liter, but heating or processing of the milk reduces the amount to around 0.35 mg. per liter. Thus both human milk and cow's milk barely meet the minimum requirement, and human milk may fall short, depending on the maternal diet. However, deficiencies are promptly corrected with the beginning of solid food supplements. While a legitimate question might be raised about the need for adding thiamine in the early months of exclusive milk feeding, nevertheless, clinical evidence of thiamine deficiency during this period is unusual.

The recommended daily allowance for riboflavin for the same periods as stated for thiamine is 0.4, 0.7, and 0.9 mg., respectively. Human milk contains an average of 0.426 mg. and cow's milk 1.57 mg. per liter. Hence the riboflavin requirements of both breast- and artificially fed infants are met from the milk alone in the first few months, and later from supplemental foods.

For niacin, the recommended daily allowance is 3, 4, and 5 mg. for the periods in infancy defined above. Human milk contains approximately 1.7 mg. of niacin per liter and

cow's milk half as much or 0.85 mg. Symptoms of deficiency do not occur because tryptophane meets part of the requirement, and milk contains adequate tryptophane.

Only in recent years has the importance of pyridoxine (B_6) come to be recognized in infant nutrition. This came about as the result of convulsions occurring in a number of infants being traced to a deficiency of pyridoxine in a milk preparation fed the infants. Cow's milk contains around 480 mmg. (0.48 mg.) of pyridoxine per liter, and human milk 110 mmg. (0.11 mg.) per liter. These amounts apparently are sufficient to protect the infant against the convulsive disorder. However, the Food and Nutrition Board of the National Research Council states that "the daily intake of vitamin B_6 should be from 1 to 2 mg.," an amount which is readily provided by ordinary mixed diets. No specific recommendation for infants is made.

On the basis that available data are insufficient, the Council has not stated a recommended dietary allowance for vitamin B_{12}. However, May and his associates[10] analyzed a number of milks commonly fed infants and found they all contain about the same, but rather small, amounts of vitamin B_{12} and folic acid compounds. They conclude that additions of vitamin B_{12} and folic acid compounds are unnecessary for normal bottle- and breast-fed infants. It is the sick and abnormal infant in whom symptoms of deficiency occur.

From the foregoing consideration, vitamin supplements for normal full-term infants may be summarized somewhat as follows: (1) for artificially fed infants, 30 mg. of ascorbic acid and 400 units (I.U.) of vitamin D should be begun early in the neonatal period—certainly by the end of the first week; (2) for breast-fed infants, 400 units of vitamin D should be begun at the same age period. Additional vitamin C may be unnecessary but does no harm; (3) for both breast-fed and bottle-fed infants, additional vitamin A and thiamine are probably unnecessary; (4) the requirement for the other vitamins that play a part in infant nutrition appears to be adequately met in the milk and solid food dietary.

Solid Food

To one who has been in the private practice of pediatrics since the early 1920's, the changing trend in the age at which solid food supplements have been introduced into the infants' diet is both interesting and almost startling. At the beginning of that period, solid foods were withheld until the latter part of the first year of life. In 1935, Marriott[6] recommended that the proper age to begin solid food additions was six months. Gradually the time has crept forward, until now many infants are having their milk diets supplemented by solid foods as early as the second and third week of life, and many more, perhaps a majority, by the beginning or middle of the second month. To the author it would appear that the present practice has developed quite independently of any consideration of nutritional needs. Rather, it would seem to have developed more as a result of experimentation and perhaps of competition between physicians and between parents. Parent insistence may have played a part also. That the digestive tracts of infants in the early weeks of life are able to tolerate these solid food additions would appear to have been demonstrated. Thus far, I am aware of no studies that have been done to determine the degree of augmentation of the renal solute load imposed by the additional minerals and protein of the solid foods such as that done by Pratt and Snyderman on milk with and without carbohydrate. Offhand, one might suppose that the small infant's water reserve might be jeopardized under certain conditions.

At any rate, there would appear to be little justification, on a purely nutritional basis, for beginning solid foods before three months of age. The infant's first nutritional need beyond that supplied by an adequate formula and vitamins is for iron and perhaps for some of the vitamins of the B complex, notably thiamine. Clearly, these can be met by medicinal administration if necessary. Iron stores are usually adequate for at least the first three or four months of life. Foods that contribute iron and thiamine are most suitable for initial use. Among these are cereals,

egg yolk, meats, vegetables, and fruits. It makes little difference which is begun first, but it is important to allow a few days to elapse between the introduction of each new food in order to permit prompt detection of any idiosyncrasy to a specific food. Egg white is best omitted until the latter months of infancy.

Some years ago it was necessary for housewives to cook and prepare foods from raw material—a time-consuming task. At present a number of commercial firms market rather complete lines of infant foods, scientifically prepared to retain essential nutritional substances such as vitamins and minerals, and at a cost within the reach of nearly every pocketbook. The foods are dispensed precooked, sterile, and in a finely divided state in small cans ready for instant use. Coarser varieties are available for older infants under the name of "chopped" or "junior" foods.

By five or six months of age the carbohydrate content of the diet from the solid food portion will be sufficient for the infant's needs. The added carbohydrate in the formula can gradually be removed and whole milk substituted from then on. Because pasteurization has little effect on curd formation, the milk should be boiled to improve its digestibility.

Summary

This article has reviewed the basic modern concepts in infant feeding. The problems relating to breast milk, cow's milk, formula calculations, protein requirement, and renal solute load are described. Mention is also made of current recommendations regarding vitamin and mineral supplementation, and consideration is given to the question of the time for the introduction of solid food.

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Nutritional Quotes

Aging, Ailing, Eating?

"The separation of the process of senescence from the chronic diseases which so often occur in old age, such as diabetes, diseases of the heart and arteries, cancer, and the arthritides, is indeed difficult and has attracted some attention. It is now common knowledge that many of these 'degenerative diseases' are related to disturbances of nutrition, and, in fact, they may be prevented in some instances and treated in others by special nutritional means. To what extent senescence itself may be altered or its onset delayed by careful attention to nutrition is quite unknown, but the problem is being attacked."
—Charles S. Davidson. *A. M. A. Archives of Internal Medicine* 94: 461, 1954.

"Nonessential" Amino Acids—Essential?

"The so-called nonessential amino acids have been known to lead to improved growth in animals when added to a diet composed exclusively of the essential amino acids. Further work emphasizing the relative nature of the nonessentiality of certain amino acids has been presented by Rose, Burr, and Sallach, who studied the effect of the omission of several nonessential amino acids in the diet. Although all of the nonessential amino acids could be manufactured to some degree with this form of dietary restriction, limitation of growth did occur, presumably owing to failure of the organism to manufacture a sufficient amount of one or more of these amino acids, even though some synthesis continued."
—Charles S. Davidson. *A. M. A. Archives of Internal Medicine* 94: 466, 1954.

Protein Problems

"Is it possible that the liberation of amino acids from proteins during alimentary digestion proceeds in the order and at the rate best adapted for their economical use in anabolic reactions? Does this account for a lower expenditure of energy when proteins are consumed than when amino acids are ingested in the free state and enter the circulation more or less simultaneously? Or do proteins contain, or carry as a contaminant, some substance which is involved in the efficient utilization of food energy or acid hydrolysis? Answers to these hypothetical questions are not available at the present time."
—W. C. Rose, M. J. Coon, and G. F. Lambert. *The Journal of Biological Chemistry* 210: 341, 1954.

Other Intrinsic Factors?

"For the present, intrinsic factor may be considered to exert a unique type of biological activity, for no other substances are known to be secreted exclusively for the enhancement of absorption of a specific vitamin from the normal diet. That there may be 'intrinsic factors' for other essential nutrients is an intriguing consideration."
—R. F. Schilling. *Federation Proceedings* 13: 769, 1954.

Blood Sugar and Blood Lipids in Diabetes

"Hyperglycemia in the human diabetic is associated with a hyperlipemia the intensity of which seems to approximate the severity of the hyperglycemia. In these hyperlipemias of diabetes mellitus, the triglyceride fraction of the blood lipids is increased the most, the cholesterol and the phosphatides slightly or not at all except in conditions of diabetic coma. When the hyperglycemic blood of a diabetic becomes normoglycemic through diet or insulin control, or both, the esterified fatty acids of the blood return to the high normal ranges. The values of the esterified fatty acids of the blood obtained during a fat tolerance test are much higher when the diabetic is hyperglycemic than the corresponding values obtained when the test is made while the blood is normoglycemic. The demonstration that hyperglycemia in a diabetic is associated with a parallel and apparently proportional hyperlipemia emphasizes that the maintenance of the blood sugar of these patients at normal levels also controls the lipid content of the blood. According to current views on the role of the lipids in the evolution of atherosclerosis, the maintenance of the blood lipids at or near a high normal range probably is significant in delaying the evolution of degenerative vascular disorders in diabetics."
—E. F. Hirsch. *Annals of Internal Medicine* 41: 550-551, 1954.

Normally Abnormal Cholesterol?

"From the viewpoint of comparative biology the high average plasma cholesterol concentration in man is pathologically high. It may then be superfluous to argue whether a relatively small increment in an already elevated blood cholesterol is significant in the production of atherosclerosis. It seems possible that beyond a threshold value, which may vary among individuals, the level of plasma cholesterol may only determine the rate at which lipids are deposited in the arterial wall. Accepting this as a working hypothesis, the possibility exists that atherosclerosis could be prevented if it were feasible to lower the plasma cholesterol level to values now regarded as subnormal."
—R. G. Langdon. *Fat Metabolism*, M & R Laboratories, 1954, p. 38.

Protein Intolerance

"The cases reported above substantiate the hypothesis that an individual intolerance to dietary protein exists in patients with cirrhosis of the liver and that exceeding this tolerance may induce or aggravate hepatic coma. . . .

"The mechanism of this protein intolerance remains obscure. A relation to ammonia metabolism seems to exist, however. Normal tissue metabolic reactions of deamination of the amino acids of ingested proteins and bacterial enzyme action on the intestinal contents provide a continuous source of ammonia. Severe hepatic cellular damage may result in a diminished ability to clear ammonia. An alternate hypothesis, in the presence of an extensive portal collateral circulation, is that portal blood containing 'ammonia' or related substances to which the brain is sensitive may occur . . . .

"The therapeutic value of a nutritious diet, adequate in protein, for patients with cirrhosis of the liver is well established. The only present indication for protein restriction is the onset or presence of impending hepatic coma or coma. The daily protein intake must depend on the individual tolerance of the patient."

—R. Schwartz, G. B. Phillips, J. E. Seegmiller, G. J. Gabuzda, and C. S. Davidson. *New England Journal of Medicine* 251: 694–696, 1954.

Liver Coma

"Considerable interest has been evidenced in the etiology of liver coma with the finding of an increased urinary excretion of glutamine and sometimes an elevated blood ammonia concentration in this condition. Moreover, impending liver coma can be induced by the administration of such substances as ammonium chloride, diammonium citrate, urea, and even increases in dietary protein in some patients. This makes it necessary that care be exercised in the administration of these substances as diuretics or in providing large quantities of protein to patients with severe liver disease."

—Charles S. Davidson. *A. M. A. Archives of Internal Medicine* 94: 462, 1954.

Food Aversions and Anxiety

"Restriction of food intake is often accompanied by multiple food prejudices born of superstition, ignorance, faulty food habits, and anxieties that have developed through association with particular foods. The role of food allergy in the development of these aversions is not clear. Experience indicates that most restrictions of specific foods are unwarranted. We have found that fat meat, fried foods, and 'gas-producing' foods are acceptable to groups of the aged. We are obliged to review many fondly held notions regarding the 'digestibility' of certain foods, and to re-evaluate what is meant by a light or convalescent diet. The criterion of what is proper food for convalescents or for the aged varies from one culture to the other, and is seen often to be a composite of medical folklore and local tradition, crystallized into current medical theory. The rigidity of prejudices and of food habits is especially marked in a small group of individuals studied who appear to have many obsessional characteristics. These people are fussy, perfectionistic, concerned with seeking security through self-imposed rituals and prohibitions. The rigidity of their attitudes is not only the product of habit but, like rigidity in earlier life, appears to be sustained by a motive, the avoidance of anxiety."

—Elias Savitsky. Psychological Factors in Nutrition of the Aged, in *Social Casework*, December 1953.

Starvation and Aversion

"When raw soybeans were fed to well-nourished human subjects . . . there was no particular difficulty encountered with their acceptability. . . . When raw soybeans were fed to human subjects in a condition of stress, in this case Americans who were Japanese prisoners of war . . . they caused nausea, vomiting, and diarrhea and were so unacceptable that these American prisoners refused to eat them even under the severest conditions of starvation. It is, of course, unknown whether these differences in the reactions to raw soybeans were due to the difference in nutritional condition of the subjects, to differences in raw soybeans fed, or to some unknown causes."

—Samuel Lepkovsky. *Advances in Food Research, Vol. IV*, Academic Press Inc., New York, 1953, pp. 125–126.

Food Acceptance and Stress

"There is better evidence with animals that foods acceptable under normal conditions became unacceptable under stress. The best example of animal experimentation along these lines concerns freshwater eels. These animals are under stress when put in salt water. In fresh water, bits of fish and dead worms are acceptable by the eels, but in salt water they will starve to death rather than eat pieces of fish or dead worms. Here a stress condition renders food which is normally acceptable completely unacceptable. Under stress these eels accept a 'tempting morsel,' a worm that wiggles. . . ."

—Samuel Lepkovsky. *Advances in Food Research, Vol. IV*, Academic Press Inc., New York, 1953, p. 126.

Nutrition Briefs

CURRENT OBSERVATIONS OF CLINICAL INTEREST

THE HUMAN requirements for vitamins are not significantly higher in arctic environments than in temperate climates.

K. Rodahl. *J. Nutrition* 53: 575, 1954.

THE INFUSION of 20 Gm. fat/hour for six hours exceeds the tolerance of an adult of average size, but 10 Gm./hour is well tolerated.

L. W. Kinsell, G. C. Cochrane, M. A. Coelho, and G. M. Fukayama. *California Med.* 81: 218, 1954.

A SATISFACTORY formula for tube feeding is composed of 1000 Gm. whole milk, 90 Gm. skim milk powder, 200 Gm. eggs, 100 Gm. dextri-maltose No. 1. 200 Gm. of 20 per cent cream, 3 Gm. salt. This supplies 2150 calories in a volume of 1500 cc., and contains 98 Gm. protein, 103 Gm. fat, 205 Gm. carbohydrate, and 104 Gm. lactose.

E. B. Smith, E. E. Wollaeger, and M. Victor. *A.M.A. Arch Int. Med.* 91: 721, 1953.

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d-AMPHETAMINE sulfate in doses of 50 to 20 mg. daily did not produce a significant or sustained rise in pressure in a group of hypertensive subjects with obesity or depression.

E. Roberts. *Am. Pract. & Dig. Treat.* 5: 606, 1954.

IDIOPATHIC hypochromic anemia may also occur in males. Such patients respond to oral iron. Poor absorption of food iron is probably an important causative factor.

J. W. B. Forshaw. *Brit. M. J.* Oct. 16, p. 908, 1954.

RECENT ADVANCES IN EXPERIMENTAL NUTRITION

RATS MADE diabetic by alloxan or pancreatectomy excreted much more inositol in their urine than normal controls. They also absorbed inositol faster than normal rats.

W. H. Daughaday and J. Larner. *J. Clin. Investigation* 33: 1075, 1954.

HYPERCHOLESTEROLEMIA of the nephrotic rat occurs in the complete absence of dietary lipids. The explanation of this endogenous hyperlipemia is probably the diminished rate of elimination of plasma lipids.

M. Friedman, R. H. Rosenman, and S. O. Byers. *J. Clin. Investigation* 33: 1103, 1954.

FOLIC ACID deficiency has been produced in dogs. The deficiency syndrome is characterized by bone marrow hypoplasia, hypochromic anemia with a tendency to microcytosis, and glossitis.

D. Afonsky. *Science* 120: 803, 1954.

SODIUM bentonite is a clay used to improve pellets in animal feeds. Evidence has accumulated showing that this substance adsorbs vitamin A or carotene in the intestine and hence is not a harmless substance.

D. H. Laughland and W. E. J. Phillips. *Canad. J. Biochem. & Physiol.* 32: 593, 1954.

DIPHTHERITIC damage and survival in guinea pigs are reported to have been favorably influenced by pyridoxine. Furthermore, in 37 human patients with severe diphtheria and resulting damage, a similar favorable effect was obtained with this vitamin, and its prophylactic use in this disease is recommended.

W. Kircher. *Intern. Ztschr. f. Vitaminforsch.* 25 (2): 175, 1954.

Money Misspent

"If all the money spent for nostrums and pseudo-scientific publications and lectures were spent for a well-chosen, varied diet, our people would be better fed. And if money were spent for adequate preventive and curative medical care rather than for shot-gun mineral and vitamin pill therapy based on self-diagnosis, thousands of Americans would be better equipped physically to enjoy the 'land of earth, sun, air, and water.'"

—A. M. Beeuwkes. *Federation Proceedings* 13: 785, 1954.

Reviews of Recent Books

Submicroscopic Morphology of Protoplasm (Second Eng. ed.) by A. Frey-Wyssling, Elsevier Press, Houston, Texas, 1954, pp. 411, $8.50.

The author is professor of General Botany at the Federal Institute of Technology at Zurich and this is the English translation of the third edition of his book.

The work is divided into three parts. The first deals with the fundamental concepts of the subject: phases in colloids, structure of crystals and gels, polarization microscopy, x-ray analysis, and electron microscopy. The second part describes the fine structure of protoplasm in general, with sections on the structure of chloroplasts, erythrocytes, and gametes. Finally, there is a description of the fine structure of such protoplasmic derivatives as chitin, cutin, keratin, and collagen.

This book is written for the non-specialist who is interested in getting an outline of the work in this field. It consists in the main of an exposition of the author's point of view with emphasis on those aspects of the subject with which the Zurich school has been especially concerned. It is therefore a monograph rather than a review.

From the point of view of the general medical reader the fact that the book has a botanical slant is a disadvantage: thus eight pages are devoted to the structure of cutinized cell walls while elastic tissue is dismissed in ten lines. As the author points out, "the chemical compounds of the cytoplasm would not be capable of accomplishing any useful work without definite positions in space," yet there is almost nothing in the book on the localization of enzymes apart from the twenty-page discussion of chlorophyll and the chloroplasts.

This is an excellent monograph and some sections of this book, such as the one on the structure of starch granules, are of special interest to nutritionists. However, the reader, interested in clinical nutrition, will find a book giving a general review of cell structure with the emphasis on mammalian cells, more comprehensible and more useful.

The print, paper, and the English of the translation show an improvement over the last English edition.

W. BEAUTYMAN

Vitamins in Nutrition and Health by A. Z. Baker, Staples Press, Inc., New York, 1954, pp. 147, $2.50.

This is a short account of the basic information on vitamins written for students of domestic science and others concerned with food planning. As an elementary discussion, it is suitable for certain college courses in nutrition or dietetics. It may also be read with profit by the interested layman. S.O.W.

Books received for review by the *American Journal of Clinical Nutrition* are acknowledged in this column. As far as practicable, those of special interest are selected, as space permits, for a more extensive review.

Fat Metabolism. A Symposium on the Clinical and Biochemical Aspects of Fat Utilization in Health and Disease, edited by V. A. Najjar, Johns Hopkins Press, Baltimore, 1954, pp. 185, $4.50.

Advances in Food Research. Vol. V, edited by E. M. Mrak and G. F. Stewart, Academic Press Inc., New York, 1954, pp. 538, $11.50.

The Vitamins: Chemistry, Physiology, Pathology, Vol. III, edited by W. H. Sebrell, Jr., and R. S. Harris, Academic Press Inc., New York, 1954, pp. 665, $16.50.

Standard Values in Nutrition and Metabolism, edited by E. C. Albritton, W. B. Saunders Co., Philadelphia, 1954, pp. 380, $6.50.

Vitamins and Hormones. Advances in Research and Applications. Vol. XII, edited by R. S. Harris, G. F. Marrian, and K. V. Thimann, Academic Press Inc., New York, 1954, pp. 305, $7.50.

Healthier Living by J. J. Schifferes, John Wiley & Sons, Inc., New York, 1955. pp. 928, $6.75.

Abstracts of Current Literature

NUTRITION AND RENAL FUNCTION

The influence of diet upon the function of the kidney in man is being emphasized by many workers. There is considerable disagreement, however, among clinicians as to exactly when one should restrict protein, and to what levels. Definite information concerning the effect of the protein intake upon renal osmotic work, renal tubular repair and function, and upon general body metabolism in renal disease is slowly accumulating. The following papers in this section deal with nutritional aspects of experimental renal disease or nephrectomy in animals.

Effect of Diets on the Anemia, Azotemia, and Survival of Bilaterally Nephrectomized Rabbits. H. Clark, P. Graham, and E. E. Muirhead. *J. Lab. & Clin. Med.* 43: 113, 1954.

Nephrectomized rabbits under apparently comparable circumstances were given various diets: "high protein-high carbohydrate"; water alone; fat emulsion (30 cal./Kg./day); 10% glucose solution (2.8 cal./Kg./day); glucose in water (30 cal./Kg./day). Total fluid intakes were similar in all groups. Control animals were also studied. Electrolyte intakes were dissimilar. The authors inferred, without presenting formal statistical evidence, that the high glucose group performed best with respect to the three criteria of anemia, survival, and hyperazotemia. Certainly the high protein (high potassium) group survived for a significantly lesser period than obtained in the other groups. The implied possibility of extrapolation to problems of acute renal insufficiency in man appears to be fraught with uncertainty. —R. TARAIL

Acute glomerulonephritis in the human has been shown to be associated with increased morbidity and a higher incidence of chronic complications when dietary protein allowances are low. Renal tubular enzyme concentrations have been shown to be reduced in protein-deficient rats. Similar analyses may be of interest in clinical material, possibly obtained by renal biopsy.

Enzyme Concentration Changes in the Kidneys of Protein- and/or Potassium-Deficient Rats. M. Iacobellis, E. Muntwyler, and G. E. Griffin. *Am. J. Physiol.* 178: 477, 1954.

Rats were rendered deficient in potassium and/or protein. The kidneys of these animals were examined for glutaminase, carbonic anhydrase, *d*-amino acid oxidase and arginase. Activities of *d*-amino acid oxidase and arginase were parallel to those of kidney nitrogen. Glutaminase and carbonic anhydrase were markedly increased in kidneys of potassium-deficient rats. These same two enzymes were decreased in kidneys of animals deficient in both potassium and protein. When potassium was deficient in the diet the total urinary acidity and ammonia excretion

were increased parallel to the increased glutaminase and carbonic anhydrase of the kidneys. When both potassium and protein deficient diets were used, total urinary acidity and ammonia excretion were reduced. —M. J. OPPENHEIMER

The production of hypertension by sustained salt administration has been demonstrated. It is of interest that in nephrectomized animals, a high protein diet is capable of maintaining hypertension.

Influence of Protein and Other Factors on Post-nephrectomy Hypertension in Rats Sustained with an Improved Method of Peritoneal Lavage. W. J. Kolff and Irvine H. Page. *Am. J. Physiol.* 178: 69, 1954.

Bilaterally nephrectomized rats were maintained by peritoneal lavage. Seventeen animals survived more than ten days. There was a steady increase in body water which depended on drinking. Body water and serum sodium changes did not produce hypertension. A high protein diet was an important cause of the hypertension.—M. J. OPPENHEIMER

Recent studies on the reabsorption of amino acids by the proximal renal tubules may shed light on the influence which various proteins and specific amino acids have upon renal tubular function.

Effect of Protein and Amino Acids Upon Renal Function in the Dog. G. W. Tank and R. C. Herrin. *Am. J. Physiol.* 178: 165, 1954.

Lean meat diets produced elevation of postabsorptive creatinine clearances in dogs. Diets with a similar amount of casein with added vitamin B did not increase postabsorptive creatinine clearances. This indicates that the effect of the meat diet depended on something else than protein as casein. Daily intake of large quantities of protein did not produce an observed proteinuria. When alanine was infused intravenously clearances of both creatinine and para-amino-hyppurate (PAH) were increased. Valanine had no significant effect in many experiments. With alanine the filtration fraction decreased in two-thirds of the cases studied. With valine however, the filtration fraction increased in 70 per cent. —M. J. OPPENHEIMER

The nephrotic syndrome, clinically and experimentally, is associated with hypoproteinemia and frequently with hypercholesterolemia. The latter has been considered a compensatory phenomenon by some observers. It is likely that a retarded rate of cholesterol destruction accounts for the elevated levels, since no increase in the rate of synthesis is noted.

Hepatic Synthesis of Cholesterol in Nephrotic Rats. S. O. Byers, M. Friedman, and R. H. Rosenman. *Am. J. Physiol.* 178: 327, 1954.

The nephrotic state was produced in rats by anti-rat kidney serum. Progressive hypercholesteremia resulted. During this time biliary cholesterol excretion was observed in order to evaluate the hepatic rate of synthesis of that substance. Hepatic synthesis of cholesterol was unchanged or diminished in the nephrotic rat. The authors suggest that the observed hypercholesteremia is neither initiated nor maintained by accelerated hepatic synthesis of cholesterol. —M. J. OPPENHEIMER

Decreased Cholinesterase Activity of Rat Kidney Following Adrenalectomy and Its Reactivation *in Vitro* by Certain Steroids. M. E. Greig and M. K. Carter. *Am. J. Physiol.* 178: 433, 1954.

Adrenalectomy reduces cholinesterase activity of rat kidney. This depressed activity could be stimulated by adrenal cortical extract, compound F, and ouabain with either acetylcholine or mecholyl substrates. DCA (desoxycorticosterone acetate) had no effect. If benzoylcholine or butyryl choline was the substrate, DCA was effective but compound F was not. Renal cholinesterase activity in control rats was unchanged or depressed by adrenal cortical extract, compound F, DCA, or ouabain.—M. J. OPPENHEIMER

HORMONES AND NUTRIENTS

The utilization of amino acids in the fabrication of certain tissue proteins involves reactions with high energy requirements. The roles of the hormones in facilitating these reactions are quite diverse and are gradually being elucidated. For example, the protein anabolism provided by the action of testosterone may involve changes in tissue concentrations of certain enzymes; while the growth hormone may shift cellular metabolism so as to spare amino acids for protein synthesis. Recently it has been shown by several workers that insulin, while it has no direct effect upon amino acid utilization, is capable of promoting the energy-producing reactions responsible for increasing the rate of uptake of amino acids by cells and facilitating their synthesis to tissue protein. Thus, insulin has been recognized as a potent growth factor.

The Effect of Insulin on Nitrogen Retention in the Hypophysectomized Rat. R. T. B. Lawrence, J. M. Salter, and C. H. Best. *Brit. M. J.* 2: 437, 1954.

In hypophysectomized rats, administration of insulin markedly increases both the absolute amount and percentage of dietary nitrogen retained. In these experiments, the relation between the amount

of insulin given and the amount of ingested nitrogen retained was approximately linear.

These results tend to confirm earlier reports from this laboratory that insulin can function as a growth hormone in the absence of pituitary secretion.—F. E. HYTTEN

The possibility that the insulin effects upon growth are mediated by epinephrine released through the stimulus of hypoglycemia is suggested by the following report. However, there are many facets of the problem requiring further study. The influence of epinephrine upon cell permeability as a factor increasing amino acid uptake may be investigated.

Further Observations on the Endocrine Regulation of Blood Amino Acids. A. C. Griffin, J. M. Luck, V. Kulakoff, and M. Mills. *J. Biol. Chem.* 209: 387, 1954.

It has been known for over twenty years that the concentrations of amino acids of the blood are markedly diminished by administration of insulin and epinephrine. Furthermore, it has been reported that insulin had no effect on the adrenodemedullated animals, although epinephrine continued to produce hypoaminoacidemia. The present study is an attempt to establish whether epinephrine alone is the effector substance responsible for lowering the level of blood amino acids.

Treatment of normal or hypophysectomized rats with epinephrine, growth hormone, or insulin resulted in a lowering of the blood amino nitrogen. The hypoaminoacidemia obtained was greatest between 2 and 4 hours after administration.

In adrenalectomized rats, growth hormone and insulin did not lower the blood amino acid nitrogen. Norepinephrine, desoxycorticosterone acetate, cortisone, corticosterone, testosterone, diethylstilbestrol, and 11-desoxy-17-hydroxycorticosterone were also without effect on the blood amino acid content of adrenalectomized rats. Since epinephrine did bring about a reduction it is reasoned that this hormone is one of the final effector substances in producing hypoaminoacidemia.

Pituitary thyrotropin reduced the blood amino acid level of both hypophysectomized and adrenalectomized rats and future studies are planned to determine the possible role of the thyroid in the regulation of blood amino acids.

The epinephrine effect on amino acid levels in the blood, though well known to the older workers in biochemistry, has not been given adequate attention in some recent papers describing studies on the effect of nutritional variables on blood and urine amino acids. The large decrease in blood amino acid obtained in man, 3 to 4 hours after insulin shock therapy, is an example of the effect of one type of stress. Since abnormal nutritional conditions may also represent stressful situations, a drop in blood amino acids observed under such conditions might be due to an effect which is secondary to the basic deficiency. —M. K. HORWITT

The well-known sparing action of carbohydrate upon nitrogen metabolism may be related to the fact that the rise in blood sugar observed after carbohydrate feeding evokes the liberation of insulin from the pancreas. The insulin thus released will indirectly result in amino acid utilization, as discussed above.

Continuous Intravenous Infusion in the Rat, and the Effect on the Islets of Langerhans of the Continuous Infusion of Glucose. B. Kinash and R. E. Haist. *Canad. J. Biochem. & Physiol.* 32: 428, 1954.

A method for continuous infusion into the rat jugular vein is described. The continuous infusion of glucose into these animals causes large increases in the weight of the islet tissue of the pancreas. Although the blood glucose levels return to normal within 24 hours, the stimulation of the islet tissue continues and appears to depend on the total amount of glucose in a given time rather than on the concentration of the sugar in the blood.—F. E. HYTTEN

ACTH, through the adrenal cortex, influences carbohydrate metabolism by increasing gluconeogenesis from the carbon moieties of the amino acids, leading to a rise in the blood sugar, and by depressing the rate of peripheral utilization of sugar. In response to a tendency of the blood sugar to rise, the islet tissue would be stimulated to produce insulin, giving rise to hyperplasia.

Effect of ACTH and of Cortisone on the Islets of Langerhans and the Pancreas in Intact and Hypophysectomized Rats. B. Kinash and R. E. Haist. *Am. J. Physiol.* 178: 441, 1954.

Daily subcutaneous injections of cortisone or ACTH increased the weights of the islets of Langerhans in hypophysectomized rats. There was only a small effect in control normal animals. Nevertheless, a continuous intravenous infusion of ACTH in these latter animals produced a much greater increase in islet size. Effects on the adrenals were much more pronounced.—M. J. OPPENHEIMER

ADVANCING KNOWLEDGE CONCERNING CHOLESTEROL

One of the important nutritional interests in the human economy is that of cholesterol, as evidenced by the vast numbers of investigations on this substance appearing in the literature. For those who desire a quick refresher course in cholesterol metabo-

lism, the following abstract and original paper are highly recommended. Data on the influence of sex and other hormones, dietary factors such as vitamins, protein, fat, sterols, and other substances currently being evaluated, are presented in this review.

Use of Food Cholesterol in the Animal Body. R. Okey. *J. Am. Dietet. A.* 30: 231, 1954.

This article reviews data concerning several factors that have been found most likely to affect the metabolism or use of food cholesterol. It also presents a lucid description of the distribution pattern in animal tissues. Thus, the liver is the central organ concerned in cholesterol metabolism. It serves as the chief site of synthesis, as a pool, and as the site of cholesterol oxidation. Cholesterol appears, in transit, in blood (80–300 mg./100 ml., normally). Cholesterol in the adrenal cortex, the sex organs, and the skin probably functions as a precursor of hormones and vitamin D. In several areas, i.e., brain, nervous tissue, muscle, and connective tissue, it may be assumed to function directly. The amount in liver may serve as a better indication of the animal's status than that in blood, but, insofar as humans are concerned, blood studies are often the only type possible. Study of the relationship of liver to blood concentration may, therefore, be important.

Factors most likely to affect the metabolism or use of food cholesterol are: (1) the percentage of dietary cholesterol; (2) the concentration, amount, and composition of solvents (fats and phospholipids) which accompany food cholesterol; (3) the concentration, amount, and amino acid make-up of the dietary protein, and the animal's need for protein; (4) the amount and type of lipotropic agents in the diet, including choline, heparin, and possibly sorbitol detergents sometimes incorporated in fat; (5) the status of the person with regard to hormonal secretion; and (6) the intake of certain vitamins.

To consider some of the above factors briefly: slight but significant increase in circulating cholesterol was noted in subjects compared directly on diets very low in cholesterol and on higher intakes. The percentage of fat in the human diet has been considered important in the type of complex in the blood. Studies are now under way on the synthesis of cholesterol from labeled acetate in rats fed isocaloric amounts of two diets, one high in fat and the other in carbohydrate.

High protein diets seem to decrease storage of liver cholesterol, but it is by no means safe to conclude that this is always the case. It has been shown that certain amino acids, e.g., methionine, tend to decrease liver cholesterol, while cystine has the opposite effect.

Several of the B vitamins seem to be concerned with cholesterol. Choline has been widely used as a dispersing agent for cholesterol. Rats deficient in pantothenic acid or in biotin do not store liver cholesterol even when given cholesterol-rich diets.

While vitamins and hormones are important in the whole picture of cholesterol metabolism, synthesis, and use, some compounds are important in cholesterol absorption. Certain plant sterols, as well as ferric chloride, have been shown to interfere with absorption in animals.

There is abundant evidence of sex differences in cholesterol accumulation in blood and tissue. Recent studies report low variable plasma cholesterol levels in the human female preceding the menopause, with a gradual rise throughout later life. In comparison, plasma cholesterol levels in males tend to be high. Values reach a peak between ages fifty and sixty, then decrease. The author indicates several areas where additional research is needed or is under way.—J. M. Smith

The influence of glycolysis in promoting lipid synthesis in hepatic tissue is established. Chaikoff's group has demonstrated that cholesterol can be synthesized within the wall of the aorta. It is likely that the vascular production of cholesterol from acetate precursors depends upon glycolysis in these tissues. A relationship between extrahepatic cholesterogenesis and atherosclerosis has not been established.

Biosynthesis and Concentration of Cholesterol by the Intact Surviving Bovine Aorta in Vitro. N. T. Werthessen, L. J. Milch, R. F. Redmond, L. I. Smith, and E. C. Smith. *Am. J. Physiol.* 178: 23, 1954.

In vitro experiments demonstrated that the aorta can concentrate cholesterol. This concentration is related to the rate at which glucose is used by the aorta. Acetate is used during the period of cholesterol concentration. The concentration of cholesterol is lowest near the heart and highest at the bifurcation of the aorta. Lipoprotein concentration of perfusates and cholesterol concentration of *in vitro* aortas were unrelated. The authors conclude from their experiments that aortas can synthesize cholesterol to approximately twice control values *in vitro*. Synthesis is preferred over removal from perfusate based on available data.—M. J. Oppenheimer

Experimental atherosclerosis resulting from cholesterol feeding has provided a useful tool in the study of this disorder. Investigators should be acutely aware of the influence of extraneous factors upon their results in such work.

Effect of Cholesterol Vehicle in Experimental Atherosclerosis. D. Kritchevsky, A. W. Moyer, W. C. Tesar, J. B. Logan, R. A. Brown, M. C. Davies, and H. R. Cox. *Am. J. Physiol.* 178: 30, 1954.

Rabbits were kept on various diets for two months. At the end of this time the animals were killed and their aortas graded (by inspection) on a 0–4 scale for atheromata. Those animals which ate rabbit food augmented with 3% cholesterol in corn oil had an average grade of 2.71. In animals on a diet augmented with 3% cholesterol in a partially hydrogenated vegetable fat the average grade was 3.71. When 3% cholesterol alone was added to the basic diet the rating averaged 3.80. Vehicles used for cholesterol in these experiments affected atheromata to different degrees only when fed with cholesterol and not when used alone.—M. J. OPPENHEIMER

Blockade of cholesterol absorption by the plant sterols, particularly sitosterol, has provided a means of lowering the blood levels in both animals and humans. Further investigations for similar substances are being pursued.

The Effect of Δ^7-Cholestenol Feeding on the Cholesterol and Lipoproteins of Rabbit Serum. R. M. Lemmon, F. T. Pierce, Jr., M. W. Biggs, M. A. Parsons, and D. Kritchevsky. *Arch. Biochem & Biophys.* 51: 161, 1954.

The cholesterol-fed rabbit develops high concentrations of low-density serum lipoproteins, a fact which some have correlated with the observation that low-density (S_f 10–20) lipoproteins are high in atherosclerosis. It had been reported that there is an unusually low quantity of Δ^7-cholestenol in human serum high in S_f 10–20 lipoproteins, so the authors ventured to determine how feeding Δ^7-cholestenol compared with cholesterol in causing the appearance of various lipoproteins. Another objective of this research was to determine if Δ^7-cholestenol acted as a precursor of cholesterol.

The results obtained from rabbit feeding experiments indicated that Δ^7-cholestenol could not be used, as had been hoped, as an antagonist or competitor to cholesterol absorption, since it produced a lipoprotein "pattern" which was qualitatively the same as that observed after feeding cholesterol. The effect of feeding both Δ^7-cholestenol and cholesterol on the levels of lipoproteins in the serum was additive. Five per cent of the serum sterols are Δ^7-cholestenol at the end of 1 week of feeding both this sterol and cholesterol, but at the end of 2 weeks the Δ^7-cholestenol falls almost to zero in spite of continued feeding of both sterols. The reason for this decrease with time is not clear.—M. K. HORWITT

The actual sites of elaboration of endogenous cholesterol and of its destruction prior to excretion into the gastrointestinal tract where it is oxidized are under study. The decrease of plasma cholesterol under conditions of reticuloendothelial blockade suggests that this tissue may not be involved in degradation of cholesterol as has been supposed.

Effect of Reticulo-Endothelial Blocking Agents on Plasma and Liver Cholesterol Levels in the Rat. L. I Rice, M. C. Schotz, J. R. Powell, and R. B. Alfin-Slater. *Am. J. Physiol.* 178: 483, 1954

High cholesterol diets increase liver cholesterol and total lipids. This is prevented by thorotrast and trypan red. These two agents do not affect liver cholesterol and lipids in rats on normal diets. Plasma cholesterol levels are decreased by these two agents in both cholesterol-fed and control animals on normal diets. The reticuloendothelial system is blockaded by these two agents.—M. J. OPPENHEIMER

Lowering of blood cholesterol levels following the administration of oxygen is a puzzling observation which may be related to an alteration in lipid metabolism in a direction away from cholesterogenesis. Several other possible explanations may be offered, providing channels for further research.

Influence of Oxygen Inhalation on Cholesterol Metabolism. R. Altschul and I. H. Herman. *Arch. Biochem. & Biophys.* 51: 308, 1954.

The treatment of cholesterol with either H_2O_2 or ultraviolet irradiation has been reported to decrease its atherogenic property. It is further claimed that most arteriosclerotic persons have a lowering of their serum cholesterol after ultraviolet irradiation.

Rabbits which were given 0.3 Gm. of cholesterol a day for 90 days and exposed to 60–65 per cent oxygen for 1–3 hours, three times weekly, had lower serum cholesterol levels than similarly fed rabbits not exposed to oxygen inhalation. The oxygen-treated rabbits are reported to have fewer atherosclerotic changes than the untreated animals.

To test this effect in humans, 19 patients were given 40 per cent oxygen at 8 L./min. for 2–4 hours. Serum cholesterol was measured before and after treatment, which lasted from 1 to 8 days, and a slight lowering of the cholesterol was claimed as a result of this treatment.—M. K. HORWITT

DIET, SERUM LIPIDS, AND ATHEROSCLEROSIS: CONTROVERSIAL ASPECTS

The metabolic regulation of lipids in serum and tissues is regarded as an important factor in the development of atherosclerosis by the adherents of the lipoprotein deposition school. On the other hand, there are those who believe that the primary lesion in this disorder is that of abnormal mucoprotein deposition in the vessel wall at points of stress, pressure, or injury. However, if the latter hypothesis is accepted as the basic factor in atherosclerosis, it is still likely that alterations in serum lipids with elevation of the S_f 12–20 lipoproteins or cholesterol levels may increase the rate of lipid deposition within the

vessel walls. The determination of the concentrations of lipoproteins has been used to predict a tendency to atherogenesis and has been found important in relation to obesity.

Relation of Fat and Caloric Intake to Atherosclerosis. J. W. Gofman, A. Tamplin, and B. Strisower. *J. Am. Dietet. A.* 30: 317, 1954.

The authors acknowledge the value of a geographic-ethnic approach in studying the genesis of human atherosclerosis. In the present paper, however, they are concerned with the relation of diet to biochemical variables important for atherosclerosis and/or coronary heart disease. It is stated that much evidence has accumulated that a disorder in the metabolic handling of lipids is ultimately associated with, and very likely responsible for, development of atherosclerosis in the human being. More specifically, such evidence implicates the high serum content of certain lipid constituents as being the *immediate* aspect of the disorder.

Ultracentrifugation has been used in Gofman's laboratory to characterize serum lipids and to show that a large series of lipoproteins serve as the serum transport vehicle for most of such serum lipids as fat, fatty acids, cholesterol, and phospholipids. In lieu of a direct measure of degree of atherosclerosis or its rate of development in the human, the authors have studied the blood picture in coronary heart disease. Thus, in a comparison of a large series of clinically well individuals of the same age, sex, and population type, lipoproteins were significantly elevated in the coronary group. In fact, two classes of lipoproteins have been distinguished, one of which is 1.75 times as important as the other in determining "coronariness." The ratio of these two fractions has been related to derive an "Atherogenic Index" or A.I. value. The usefulness of this ratio in predicting coronary disease is illustrated by means of a probability curve for men 40–49 years of age where A.I. values are related to absolute probability of development of clinical disease in any one year. The probability curve is based on data on deaths/100,000 men 40–45 years per year supplied by the Metropolitan Life Insurance Company. The authors emphasize that as the incidence of deaths per 100,000 per year changes, the entire curve would shift to higher or lower values, but the relative positions of points on the curve and the general shape of the curve would be uninfluenced. The predictive value of this curve should be useful in considering A.I. values in relation to such possibilities as dietary prophylaxis or therapy of atherosclerotic coronary heart disease.

The authors believe recent mortality data of Dublin and Marks and data on incidence of excessive coronary artery atherosclerosis in overweight persons on autopsy lead to a conclusion that a positive association exists between coronary disease and overweight. The information does not tell the mechanism by which overweight operates in producing excessive morbidity and mortality from coronary heart disease.

By means of A.I. values, as compared with the probability curve mentioned above, the authors estimate that men 40–49 years of age and 40 per cent overweight have a probability of coronary disease 1.7 times that of men the same age whose weight does not exceed the "ideal weight."

It is emphasized that such data are "average values," as underweight persons with elevated A.I. values are not rare, and many overweight persons have low or moderate A.I. values. Thus, an intelligent approach to individual prevention and therapy *via* overweight correction would be first to classify obese persons as to A.I. levels.

It is recognized by these authors that (1) excessive fat intake, (2) calories *per se*, or (3) some "metabolic failure of the obese state" may account for elevated A.I. values in overweight people. It was found that lowering of fat intake at an isocaloric level led to lowered A.I. values in humans. Thus elevated average A.I. values characteristic of obese persons could be, at least in part, the result of excessive fat intake. There has been no valid evidence that calories *per se* affect lipoprotein values, but further, better, controlled studies are needed for an unequivocal answer. The possibility of metabolic disturbances is essentially unevaluated.

Certain geographic studies have implicated high animal fat intake in association with atherosclerosis and coronary disease. Experimental evidence is cited to show that in relatively short-term experiments in humans (days, weeks, and months) fat of both animal and vegetable origin must be restricted to lower serum lipoproteins and that vegetable fat alone can raise lipoproteins after they have been lowered by dietary means. The concern over animal fat is the result of its cholesterol content, which in animal experiments is implicated with elevated lipoproteins and atherosclerosis production. To date, there are no good data on humans to show whether high fat, high cholesterol intake may elevate serum lipoproteins more than high fat, cholesterol-free diets. —J. M. Smith

Geographic-economic studies in nutrition have been conducted in various areas of the world. The relationship between dietary intake of fat, the serum cholesterol levels, and the incidence of atherosclerosis has been evaluated in some of the previous reports. It is of interest that, in general, the serum cholesterol is independent of dietary fat intake; however, in middle-aged men on high fat diets the serum cholesterol rises as does the incidence of coronary artery disease.

Serum Cholesterol and the Diet in Clinically Healthy Men at Slough near London. A. Keys and M. H. Keys. *Brit. J. Nutrition* 8: 138, 1954.

Clinically healthy men, from 40 to 55 years old, at Slough, England, subsisting on a diet in which fats contributed an average of 35.4 per cent of the calories were compared with men of the same age in Minnesota, and in Naples, Italy, in whose diets fats provided about 40 and 20 per cent of the calories, respectively. In all three groups, proteins provided from 12 to 13 per cent of total calories. Compared with the Italian and American groups, the Slough men were thin, but their weights corresponded closely to the data for relative body-weights from larger samples of Englishmen.

The mean total cholesterol concentration in the Slough men was not significantly different from that in the Minnesotans, but was definitely higher than in the Neapolitans. In none of the groups of men was there any significant relationship between relative body-weight and the serum cholesterol concentration. Evidence is presented to show that the dietary intake of cholesterol of such men as those studied at Slough is about half that of men in Minnesota, and that in man the serum concentration of cholesterol is unrelated to the dietary intake. The data from Slough, combined with other evidence from England, the United States, Denmark, Italy, and Spain, show that in healthy young men the serum cholesterol value is substantially independent of the diet, but that in middle-aged men on relatively high fat diets, as in England and the United States, the serum cholesterol concentration is significantly higher than in men on relatively low fat diets, as in Italy and Spain.—B. SURE

An intriguing theory concerning the pathogenesis of atherosclerosis has been published in England. The concept presented is that of the formation of gradually increasing superficial thrombi involving the intima and producing mucoprotein deposits.

Diet and Coronary Disease. J. B. Duguid. *Lancet* 1: 891, 1954.

The author questions the foundations of the hypothesis that atherosclerosis is related to diet. He believes that confusion arises from misinterpretation of the histological picture and considerable evidence is presented of the natural history of arterial lesions. The fats which appear in atherosclerotic lesions may be due to circulating fat being incorporated into thrombi formed for other reasons, and are, therefore, a secondary phenomenon. The cholesterol experiments, on which he believes much of the diet hypothesis hinges, are said to be fallacious. The lesions are only produced by amounts of cholesterol far in excess of the normal human diet and these lesions are, in any case, not of the kind which leads to vascular insufficiency.

Professor Duguid takes, perhaps, rather a narrow view of the theory, since the relationship between a high fat diet and the incidence of coronary disease would appear, from Keys' data, to be undoubted; in any case, cholesterol is not necessarily the villain of the piece, but a good index of the level of lipemia. —F. E. HYTTEN

Permeability factors may be of significance in atherosclerosis, since hypercholesterolemia does not lead to formation of the lesion in the presence of cortisone. On the other hand, hyaluronidase, which increases tissue permeability, promotes the formation of atheromata, according to Seifter.

Adrenal Cortex, Lipid Metabolism, and Atherosclerosis; Experimental Studies in the Rabbit. D. Adlersberg, L. E. Schaefer, and C-I Wang. *Science* 120: 319, 1954.

The combination of cortisone or hydrocortisone administration, in addition to cholesterol feeding, in rabbits, results in a considerable elevation of plasma lipid fractions—definitely higher than those seen on cholesterol feeding alone. Discontinuation of corticosteroid administration but continuation of cholesterol feeding resulted in a return of the lipid level to that of cholesterol feeding alone. Corticotropin (ACTH) was less effective than the steroids.

Atherogenesis and the deposition of cholesterol in other tissues was inhibited by this combination in spite of the extreme hypercholesterolemia. The authors suggest that corticosteroids diminish tissue permeability of lipids.—S. O. WAIFE

Further evidence supporting the influence of dietary fat intake upon serum cholesterol and atherosclerosis is presented in the following reports dealing with the African Bantu. These data corroborate the observations of Keys.

Fat Intake, Serum Cholesterol Concentration, and Atherosclerosis in the South African Bantu. Part I. Low Fat Intake and the Age Trend of Serum Cholesterol Concentration in the South African Bantu. A. R. P. Walker and U. B. Arvidsson. *J. Clin. Investigation* 33: 1358, 1954.

The South African Bantu habitually eats a diet containing about half as much fat as the American diet. This study reports a survey of blood cholesterol levels among 218 Bantu subjects of all ages.

Up to age 40 there was no significant difference between mean values for the Bantus and those reported by Keys from Minnesota. After this age, however, the serum cholesterol levels are significantly lower among the Bantus.

The urban native, consuming a European diet (higher in fat), had a significantly higher mean cholesterol level than the rural Bantu. Other factors, such as caloric intake, liver disease, and racial stock,

seem to have little importance in influencing serum cholesterol levels. Probably the low fat diet was the most significant factor, although the high fiber content of the diet may also bear some responsibility.—S. O. WAIFE

Fat Intake, Serum Cholesterol Concentration, and Atherosclerosis in the South African Bantu. Part II. Atherosclerosis and Coronary Artery Disease. J. Higginson and W. J. Pepler. *J. Clin. Investigation* 33: 1366, 1954.

Several previous reports had noted the infrequency of coronary artery disease among certain African groups. A review of postmortem material at a non-European hospital in Johannesburg, South Africa, revealed a lower incidence of severe atherosclerosis than in Danish or American hospital populations. The population which this hospital serves is habituated to a low fat, high residue diet.—S. O. WAIFE

DIETARY EVALUATION AND SURVEYS

Much of our information concerning nutritional requirements is derived from surveys conducted on various population groups. The observations conducted by organizations equipped to evaluate the dietary habits, health, and activities of people in differing age groups, occupations, and clinical states comprise a significant contribution to our understanding of the practical problems in nutrition, serving as a complement and check to intricate metabolic studies. An examination of methods employed by nutritionists is necessary for a more complete picture.

Group Method of Food Inventory vs. Individual Study Method of Weighed Food Intake. M. E. Carroll, M. A. Wharton, B. L. Anderson, and E. C. Brown. *J. Am. Dietet. A.* 28: 1147, 1952.

Dietary data collected in an Ohio institution were compared by nutritive evaluation of diets recorded by the group method of food inventory or by weighed intakes of individual portions. In the *group method,* inventories of food available at the beginning and end of a month supplemented with records of foods purchased in the interim were prepared for four kitchens. The content of several nutrients was estimated from tables of values listed for individual foods or from tables in which foods with similar nutrients are grouped. During the same month, representative food servings were weighed during six weekdays, with random selection of days. The weighed dietaries were calculated for calories, protein, calcium, iron, vitamin A, thiamine, riboflavin, niacin, and ascorbic acid from tables listing individual food items. These weighed food portions were analyzed chemically for protein, thiamine, and riboflavin content.

Protein and calcium content of diets did not differ greatly by any of the methods studied. The individual study method on weighed portions calculated by food tables gave higher values for all nutrients than the group method. Calculation of food inventory data by food groups gave greater values for iron, thiamine, riboflavin, and niacin than when these data were calculated by individual food values. Thiamine content of weighed food portions was 5 per cent lower by chemical analyses than by calculations from tables of individual food values (this difference was significant for only one of the four kitchens). Analyzed riboflavin values were 15 per cent greater than calculated, with a significant difference for two kitchens.

The authors demonstrated a significant correlation between content of protein and thiamine, protein and riboflavin, and thiamine and riboflavin in the dietaries studied. They emphasize that further study should be made of these relationships in many types of diets before a generalization can be reached concerning dietaries of the general population.—J. M. SMITH

Weekly Variation in Nutrient Intake of Young Adults. C. M. Young, R. E. Franklin, W. D. Foster, B. F. Steele. *J. Am. Dietet. A.* 29: 459, 1953.

It is the purpose of this paper to report a study of the weekly variation in nutrient intake of a group of young adults at one season of the year. Eighteen adults, 23 to 50 years of age, eating mostly in their own homes, recorded dietary intakes as estimated or measured food portions for 28 days. The record of each individual was calculated in terms of average weekly and average 28-day nutritive value. Records were calculated for calories, protein, calcium, phosphorus, iron, vitamin A, thiamine, riboflavin, niacin, and ascorbic acid by Babcock's simplification of the long method.

On both an individual and group basis, a study was made of the variation in weekly nutrient intake and of changes in evaluation relative to the National Research Council Recommended Dietary Allowances, which would have occurred if one-week records had been used instead of 22-day records. Variation in the 28-day records was also partitioned according to days, weeks, and subjects.

When the average intakes for the group were examined, one week was representative of the total period. To a less pronounced degree this held for individuals for calories, protein, phosphorus, iron, vitamin A, thiamine, riboflavin, and niacin; it did not hold for calcium and ascorbic acid.

As an estimate of average nutrient intake for a group, a seven-day record was adequate. For an individual, it was felt that the observation period should exceed seven days for most subjects.

This article presents an excellent review of prior work which attempted to solve the problem of the length of time required to record a representative picture of customary diet habits.—J. M. SMITH

A Fallacy in the Analysis of Nutritional Requirement Data. H. J. Almquist. *Arch. Biochem. & Biophys.* 50: 503, 1954.

This communication is a "letter to the editor" which proposes that errors arising from a misinterpretation of certain statistical principles have resulted in conclusions which have caused the nutritional requirements of animals to be subjected to a tendency toward underestimation. Dr. Almquist feels that the ordinary use of statistical tests in nutritional studies does not take into account the principle of diminishing returns when the biological response is plotted against the high intake levels of a given nutritional factor. He suggests that the error can be avoided by plotting the biological response against the log of the concentration of the nutritional factor being studied.—M. K. HORWITT

In many of the nutritional surveys conducted outside the United States, the dietary allowances recommended here are regarded as being excessive with respect to vitamin A and ascorbic acid, as in the following paper. Although the U. S. National Research Council's Recommended Allowances provide a wide margin of safety, it must be recalled that such allowances are not always practicable and that the organism is capable of adaptation to various levels of intake of specific food factors.

Nutrition Among Lower Income Classes in Finland. O. Turpeinen. *Ann. Med. Int. Fenniae* 42: 75, 1953.

In a nutritional survey carried out among lower income groups in Finland during 1936–37, the diets of 88 families comprising 619 persons in 24 different localities were studied by inquiry during a period of 5 to 10 days. Two-thirds of the group were under 20 years of age. In comparison to other human dietary studies, the dietary of the Finns was characterized mainly by a high consumption of milk products, relatively high consumption of cereals and potatoes, and a relatively low consumption of sugar, meat, and vegetable fats. There was an almost complete absence of fruits. The diet was found to be poor in vitamin A, but relatively rich in protein, calcium, phosphorus, iron, and thiamine. The most significant finding was that the mean vitamin A intake was only about half the recommended dietary allowances of the U. S. National Research Council. The author feels that the usually recommended allowance for ascorbic acid is excessive and believes that a daily intake of about 25 to 30 mg. is more realistic. With this standard, the ascorbic acid intake of the studied population was adequate.—S. O. WAIFE

ENERGY REQUIREMENTS

The determination of the caloric requirements of an individual requires knowledge of the basal metabolism, specific dynamic action of foods, temperature variations, physical activity, and growth. It is essential that a close approximation of the energy requirements be established in nutritional endeavors, since the efficiency of the individual and his total metabolism depend largely upon this phase of his dietary intake. The most variable factors influencing the caloric requirements are activity and temperature changes, so that further studies dealing with these are of interest. Such information is vital not only in relation to military medicine, but also in the management of chronic disease states, in diabetes, and in evaluating the requirements for various types of labor.

The Nutrition of Male Industrial Workers with Particular Reference to Intake and Expenditure of Calories. E. R. Bransby. *Brit. J. Nutrition* 8: 100, 1954.

A previous pilot survey had suggested that calorie intake did not, on the average, vary between groups of men doing work of different degrees of activity. A further survey was, therefore, made early in 1952 of the diet and activities of men working in a number of factories in Slough on jobs of different degrees of heaviness. The number medically examined was 174, and information on diet and out-of-factory activities was collected for 152 of them. Results on 137 of the men who could reliably be presumed fit were used for a study of calorie intakes and expenditures. For the group of 152 men, the average daily intake was 3549 cal., 109 Gm. protein; 138 Gm. fat; 435 Gm. carbohydrate; 21 mg. iron; 1.3 Gm. calcium; 4171 I.U. vitamin A; 1.7 mg. thiamine; 14 mg. nicotinic acid; 1.8 mg. riboflavin; and 42 mg. ascorbic acid.

The average daily calorie intake of men doing light work was about 600 cal./day less than that of men doing heavy work. The previous failure to differentiate between intakes of calories was thus not confirmed. The estimated calorie expenditure per minute, excluding what was required for basal metabolism, was on an average 2.2 cal. for light work, 2.7 for light to medium work, 2.6 for medium heavy work, and 3.8 for heavy work. For the whole sample it was estimated that the percentage of the calorie intake spent on basal metabolism and specific dynamic action was 46, on out-of-factory activities 22, and on work 32. The percentage spent on work varied from 26 for men doing light work to 41 for men doing heavy work. The tentative nature of these estimates is emphasized.

On an average, 27.9 per cent of the whole day was spent at work and 35.4 per cent in sleeping. Of the remainder, 21.6 per cent was on an average spent in sitting, 3.8 per cent in dressing, 3.8 per cent in domestic work, and about 2 per cent or less in each of the other activities listed in a table in the paper. Of the energy spent on out-of-factory activities, 19.8 per

cent was spent in sitting, 17.4 per cent in dressing, 20.6 per cent in cycling, 16.7 per cent in domestic work, and 13.1 per cent in walking, with only small proportions in other activities.—B. SURE

The Food Intake and Energy Expenditure of Cadets in Training. E. M. Widdowson, O. G. Edholm, and R. A. McCance. *Brit. J. Nutrition* 8: 147, 1954.

If groups of adults are maintaining a steady average weight, it may be assumed that their caloric intake is meeting their requirements. Standard growth curves have been established for children and, if a group of them is growing satisfactorily, their nutritional requirements are probably being covered by the food provided for them. It is not, however, so easy to be sure of this at an age when growth may or may not be complete and when a gain in weight might be desirable owing to muscular development even if growth in height has ceased. This was the problem facing those responsible for the health of a large number of young men aged 18.5 to 20 years at one of the training establishments for the armed forces.

The energy expenditure of 77 cadets in a training establishment has been estimated and found to average 3420 cal./day. This is equivalent to published figures for the energy expenditure of men engaged in moderate work. The cadets spent $9^1/_4$ hours a day sitting, some of it at lectures, and $8^1/_2$ in bed. Dressing and cleaning uniform occupied more of their time and energy than sport or military training. The supplies issued to the establishment provided 3714 cal./cadet/day. Of these the cadets took only 68 per cent. Unused bread accounted for a large part of the discrepancy, for the cadets ate only 6.4 oz. out of the 15 oz. of their ration. The plate waste came to 7.7 per cent of the calories in the food served. The cadets then bought at the canteen and in restaurants outside food that provided them with approximately the same number of calories as the portion of their ration not taken up. By refusing their allowance of bread and buying cakes instead, they obtained more fat but less protein than their ration supplied.—B. SURE

Regulation of Food Consumption by Caloric Value of the Ration in Rats Exposed to Cold. E. A. Sellers, R. W. You, and N. M. Moffat. *Am. J. Physiol.* 177: 367, 1954.

Rats were kept at an environment of 1.5° C. They were given a diet containing a constant amount of protein but with variable (5–44 per cent) content of fat. Those animals getting a high fat diet ingested more food than those with a low fat intake. In each case the caloric values were the same on both high and low fat diets. Protein intake and nitrogen excretion were less on high fat diets. This was so because fat has a higher caloric value per unit of weight, with the result that the necessary calories could be obtained from a smaller food intake and less protein was taken in when fats were high. Growth rate was less than that of controls at room temperature no matter what diet was taken.—M. J. OPPENHEIMER

LIPOIC (THIOCTIC) ACID*

***Symposium on Metabolic Role of Lipoic Acid (Thioctic Acid).** Chairman: G. W. Kidder. *Fed. Proc.* 13: 695 ff., 1954.

The first recognition of thioctic acid in nature was the observation in 1941 that ciliated protozoan, Tetrahymena, could be grown in a casein medium only when supplemented with crude fractions of natural materials. This factor was required when all of the known vitamins and growth factors were supplied. Later it was shown that acetate could be replaced in L. casei *culture medium by yeast extract and that* Str. feacalis *failed to oxidize pyruvate unless a yeast factor were present. In 1949, the growth factor required for the tetrahymena was termed "protogen" and was found to be identical with the "acetate replacing factor" and the "pyruvate oxidation factor." In 1951, the growth factor was crystallized and the name (alpha-) lipoic acid was proposed. Later, when its chemical structure became known, the term thioctic acid was adopted as being descriptive of the compound.*

Chemical and Photochemical Reactions of Thioctic Acid and Related Disulfides. M. Calvin. *Fed. Proc.* 13: 697, 1954.

The author establishes the connections between the photosynthesis cycle and the respiratory or citric acid cycle in algae. Radioactive carbon incorporated in phosphoglyceric acid in the absence of light is shown to enter into the compounds of the citric acid cycle, while in the presence of light the carbon label appears in the members of the photosynthesis cycle within the algae preparation. The reactions required to bring the 3-carbon groups into the citric acid cycle involve the "pyruvic acid oxidase factor" (thioctic acid) which is a 5-carbon cyclic disulfide. Pyruvic acid is decarboxylated to form acetyl-thioctic acid, giving off CO_2 in conjunction with the action of thiamine pyrophosphate at this level. The active acetyl undergoes a thiol exchange with coenzyme A which brings the 2-carbon fragment into the citric acid cycle. It was suggested that the disulfide linkage of thioctic acid is reduced to the dithiol form in the presence of light, so that the compound is incapable of oxidizing pyruvic acid to acetyl thioctic and CO_2. In the presence of the reducing power generated by light, the photosynthesis cycle would be favored. The disulfide chemistry is presented in considerable detail.—C. R. SHUMAN

Oxidative and Transfer Reactions of Lipoic Acid. I. E. Gunsalus. *Fed. Proc.* 13: 715, 1954.

Lipoic acid has been shown to undergo reduction with acylation, acyl transfer reactions, or through an oxidative reaction. These reactions are reversible, although the actual mechanisms and equilibrium constants in reductive and reoxidative phases are not clear. The reactions described operate in the oxidative decarboxylations of the alpha-keto acids in bacterial and animal tissue systems. The recognized steps in the oxidation of keto-acids involve: (1) thiamine as the initial aldehyde acceptor and decarboxylating coenzyme; (2) lipoic acid as intermediate acyl acceptor; and (3) coenzyme A as the ultimate acyl acceptor. Oxidized lipoic acid is regenerated following electron transfer to DPN.—C. R. SHUMAN

Enzymatic Synthesis of a Lipoic Acid Coenzyme. L. J. Reed and B. G. DeBusk. *Fed. Proc.* 13: 723, 1954.

Lipoic acid functions in the oxidative decarboxylation of alpha-keto acids as a thiamine pyrophosphate-lipoic acid complex (LTPP). The lipoic acid-thiamine linkage has been thought to occur as an amide between the carboxyl group of the acid and the primary amino group of the thiamine pyrophosphate molecule. The coenzyme has been obtained by enzymatic synthesis by lipoic acid conjugase. In addition, it has been prepared by chemical synthesis and was obtained from yeast. The conjugate system can synthesize the coenzyme if supplied coenzyme A, ATP, thiamine pyrophosphate, and lipoic acid.—C. R. SHUMAN

VITAMINS B_T AND T

The appellation "vitamin T" has been applied to a growth factor found in an extract prepared from termites. There appears to be no obvious relationship between this factor and that which has been designated as B_T (carnitine). Since they are both new substances in the vitamin galaxy, the following abstracts are presented together. The question as to whether or not these substances are actually vitamins or, indeed, play any role in human metabolism, remains to be answered. However, the remarkable qualities attributed to vitamin T make it of some interest, and confirmation is clearly needed. The wide distribution of vitamin B_T suggests that further study of this factor would be desirable, and it is being pursued in several centers.

Vitamin T. R. Martinez Callen. *Rev. españ. de Pediatria* 6: 363, 1952.

"Vitamin T" was discovered in 1945, during studies on ants and termites. Investigating the difference between the ordinary "workers" and the "soldiers" (individuals with bigger heads and large mandibles) in the species *Calloternes flavicollis* and *Reticulotermes lucifrugas*, Goetsch discovered that there was no difference in the eggs which produced the two types. The emergence of "soldiers" depended on a more abundant food supply, especially of proteins, furnished at a certain stage in the development of the larva. He obtained from the termites an extract which he called "Termitine"—the precursor of "vitamin T"—which, when administered to the larva, transformed them into soldiers. Equivalent transformations were achieved by the administration of the substance to other insects, and these effects were not obtainable with any of the classical vitamins. It was later discovered that the T substance is not produced by the termite organism, but furnished by vegetal substances lacking in chlorophyll: fungi and yeasts. Among the fungi, the T substance is found in Hypomyces, penicillium, and mucor; among yeasts, in the Torula and Saccaromyces. The "T complex" obtained from yeasts includes hypomycin, penicillin, torulitin, etc. However, the T substance is not found in yeast in the active form, but in combination with antagonists which counteract its effects. Like ergosterine, it must be activated.

Biochemically, the T substance has not yet been completely identified. It is not a simple substance, but a complex made up of various products, soluble in water and in dilute alcohol, and heat-resistant up to a temperature of 120°. It is related to the B complex, with which it is found in nature, though neither the B complex nor any one of its several members reproduces the effects of T preparations. The effectiveness of "vitamin T" is enhanced by the presence of other active substances; hence commercial T preparations include other vitamins, especially B_1, B_2, and B_{12}. Experiments in frogs have shown that to be most effective, T requires the presence of additional factors, amino acids as well as vitamins, though none of these factors, with or without added B complex, is capable of producing the effects of the T substance.

Successful animal experiments followed those on insects and frogs, and experiments in man were first performed by Heyn, followed by Nuhsbaumer, Glanzmann, and Torres Marty. Their results seem to show that while "vitamin T" is not absolutely necessary to human beings, its administration produces favorable organic and psychic effects.

The effectiveness of this substance is especially notable in cases where it is desired to accelerate cellular growth. It has been used successfully in burns and frostbite, where even local application produces rapid healing. Its administration should always be preceded by antibacterial therapy, and it should be applied only to clean lesions.

It is in pediatrics, however, that "vitamin T" has been used with the greatest success—particularly in new-born infants and "atrophic" children. It has also been said to be remarkably effective in celiac disease;

the osteoporosis associated with this condition, and which is unaffected by vitamin D, has been cured by the concomitant administration of "vitamin T." The same synergism between vitamin D and the T substance has been observed in rickets: the fat-soluble vitamins, especially, are potentiated by "vitamin T."

The suggestion is made that the effectiveness of "vitamin T" should be most marked in influencing embryonic development, and that it would be logical to try the effects of its administration to pregnant women. The growth- and appetitite-stimulating effects of "vitamin T" have not been accompanied by toxic effects in the cases reported.—C.-J. Howell

Infantile Anorexia and Vitamin T. M. Ruiz Ubeda. *Rev. españ. de Pediatria* 8: 303, 1952.

The author reviews briefly the history of vitamin T. Though its chemical composition is still unknown, "vitamin T" seems to be a potent biocatalyst of growth and assimilation, and to be somehow related to vitamin B_{12}, desoxyribose, the *Lactobacillus bulgaris* factor, folinic and folic acids, and to the F factor.

"Vitamin T" has proved to be an effective anti-anorectic and to increase digestive tolerance in premature infants whose weight was stationary. Its effectiveness has been reported in rickets, osteoporosis, and other conditions; in celiac disease its action has been remarkable—serum protein levels increasing in some cases from 5.33 to 7.24 Gm. per 100 ml. in only 3 weeks of treatment. Indeed, the list of conditions in which the beneficial effect of "vitamin T" has been reported is long; it includes paralysis and paresis, poliomyelitis, acute pseudo-paralytic myasthenia, muscular dystrophy, pituitary cachexia (even, paradoxically, obesity of the same etiology), sexual neurasthenia, abortions, dermatoses such as infantile eczema, psoriasis, burns, allergic dermatitis, etc.

Goetsch recommends 500 units for infants up to 6 months, 750 units for those from 6 months to 3 years, and 1000 units for those over 3 years. These amounts are given, in divided doses, about 20 minutes before meals. After 20 days, treatment is suspended for 10 days, then resumed for 20 more, and so on.

This was the schedule followed by the author in 4 cases of infantile anorexia which had resisted other forms of treatment (eupeptics, multiple and single vitamins, liver, etc.). In 3 of the 4 cases, "vitamin T" gave excellent results: the children recovered their appetites and gained weight, and the symptoms which had in some cases accompanied the anorexia (vomiting, abdominal pains) disappeared. In the fourth case, the anorexia persisted, though the child gained 250 Gm. and the anemia which had been present before "vitamin T" treatment, disappeared. The author concludes that "vitamin T" is highly beneficial in overcoming anorexia in children.—C.-J. Howell

The Distribution of Vitamin B_T (Carnitine) Throughout the Animal Kingdom. G. Fraenkel. *Arch. Biochem. & Biophys.* 50: 486, 1954.

Carnitine, which is required by the mealworm and some other beetle larvae, was originally discovered in muscle extract. It has been suggested that since the mammalian muscle constituted a particularly rich source of carnitine, vitamin B_T had some function in the metabolism of muscle. To test this hypothesis, a variety of muscles were analyzed for their carnitine content.

A survey of the carnitine levels of whole animals or of single organs from a variety of marine invertebrates and vertebrates showed that the carnitine content varied enormously between different animals and tissues and showed no correlation to the phylogenetic relationships. The carnitine content of muscles from a vitamin E-deficient rabbit and of a dystrophic calf appeared to be quite normal. Nor did the breast muscle of a bat which has a high metabolic activity differ from other mammalian muscle in its carnitine content. No evidence was obtained to show whether or how carnitine functions in muscle. However, since it occurs in all forms of animal life, it must be considered to have some metabolic function.—M. K. Horwitt

ITEMS OF GENERAL INTEREST

The Flavour of Porridge. T. Moran, J. B. Hutchinson, and J. Thomlinson. *Nature* 174: 458, 1954.

It is considered that the traditional "nutty" flavour of porridge is often lacking today, and may account for the declining popularity of this cereal.

The flavour depends on the method of kilning; optimum conditions correspond to the gentle drying of the oats down to about 8 per cent water, followed by a short toasting in air at about 150° C. The flavour is characteristic of oats and also groats, but could not be produced by subjecting wheat to the same treatment. The substances responsible for the flavour have not been identified.—F. E. Hytten

Multiple Factors in Thirst. E. F. Adolph, J. P. Barker, and P. A. Hoy. *Am. J. Physiol.* 178: 538, 1954.

Depletion of body water or an excess in the body of a solute which is hypertonic to blood plasma is necessary to induce drinking in these experiments. Drinking was induced when various doses of salt or urea were added by three routes and subsequently

absorbed into the circulation. Water drunk was in an amount sufficient to dilute the salt to 0.4 mEq./L. and excrete it in that concentration. The amount of water taken was never enough to make an isotonic salt solution using body fluids as a control reference. Many things partially inhibited drinking: severe restraint, filling the stomach, administration of water by other routes, injection of pitressin, cocainization of the mouth, high concentration of solutes in drinking water. Dilute sodium chloride solutions as drinking water increased oral fluid intake. Weight was lost during water deprivation.—M. J. OPPENHEIMER

Ingested Sodium Glutamate and Plasma Levels of Glutamic Acid. W. A. Himwich and I. M. Petersen. *J. Appl. Physiol.* 7: 196, 1954.

Sodium glutamate has been used commercially as a "taste-improver." This paper reports some observations on this popular substance. Glutamic acid varied widely in basal conditions and after a placebo. Ingestion of 45 grams of glutamic acid daily produced no effect on basal plasma level of glutamic acid or glutamine. Effects of single doses of sodium glutamate upon plasma levels varied with the dose and with the individual.—M. J. OPPENHEIMER